PARASITOLOGY

(Founded by G. H. F. Nuttall, F.R.S.)

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CONTENTS

Nos. 1, 2 (January, 1946)

PAGE

Lees, A. D. The water balance in *Ixodes ricinus* L. and certain other species of ticks. (With 8 Figures in the Text) 1

Smith, Kenneth M. *Tobacco rosette*: a complex virus disease. (With Plates I and II) . 21

Smith, Kenneth M. and Lea, D. E. The transmission of plant viruses by aphides. (With 7 Figures in the Text) 25

Rees, Gwendolen. A record of the nematode parasites of fishes from the Porcupine Bank, Irish Atlantic Slope and Irish Sea 38

Radford, Charles D. Notes on *Trombicula deliensis* Walch, 1923 (Acarina; Trombidiidae), with description of the adult. (With 9 Figures in the Text) 42

Radford, Charles D. Larval and nymphal mites (Acarina; Trombiculidae) from Ceylon and the Maldive Islands. (With 29 Figures in the Text) 46

Lewis, E. Aneurin. Nairobi sheep disease: the survival of the virus in the tick *Rhipicephalus appendiculatus*. (With 6 Figures in the Text) 55

Lewis, E. Aneurin, Piercy, S. E. and Wiley, A. J. *Rhipicephalus neavei* Warburton, 1912 as a vector of East Coast Fever. (With 4 Figures in the Text) 60

Phillips, Nada E. and Mackinnon, Doris L. Observations on a monocystid gregarine, *Apolocystis elongata* n.sp., in the seminal vesicles of *Eisenia foetida* (Sav.). (With 45 Figures in the Text) 65

Milne, A. The ecology of the sheep tick, *Ixodes ricinus* L. Distribution of the tick on hill pasture. (With 2 Figures in the Text) 75

Robinson, G. G. The biology of *Ornithodorus delanoei acinus* Whittick, 1938 82

Bishop, Ann and Gilchrist, Barbara M. Experiments upon the feeding of *Aëdes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria. (With 2 Figures in the Text) 85

Chang, Shih Lu. Studies on *Entamoeba histolytica*. IV. The relation of oxidation-reduction potentials to the growth, encystation and excystation of *Entamoeba histolytica* in culture. (With 1 Figure and 9 Graphs in the Text) 101

Nos. 3, 4 (August, 1946)

Fonseca, F., Cambournac, F. J. C., Pinto, M. R., Pereira, J. M. and Cunha, A. Studies on the exo-erythrocytic cycle of malaria. (With Plate III) 114

Wilson, S. G. Seasonal occurrence of Ixodidae on cattle in Northern Province, Nyasaland. (With 2 Figures in the Text) 118

PAGE

SMITH, KENNETH M. Tomato black-ring: a new virus disease. (With Plates IV and V) . 126

SMITH, KENNETH M. The transmission of a plant virus complex by aphides . . . 131

ROONWAL, M. L. and NATARAJ, S. On two species of sucking lice (Anoplura, Haematopinidae) from the common North Indian five-striped squirrel (*Funambulus pennanti pennanti* Wr.). (With 1 Figure in the Text) 135

WATSON, J. M. Studies on the morphology and bionomics of a little-known holotrichous ciliate—*Balantiophorus minutus* Schew. Part III. Coprophilic habits and the effect of urine 138

ARTHUR, D. R. and EDWARDS, E. E. A note on an unusual male tick from Glamorgan. (With 6 Figures in the Text) 152

ARTHUR, D. R. The feeding mechanism of *Ixodes ricinus* L. (With 15 Figures in the Text) . 154

REES, GWENDOLEN. The anatomy of *Phyllobothrium dohrnii* (Oerley) from *Hexanchus griseus* (Gmelin). (With 20 Figures in the Text) 163

LEES, A. D. Chloride regulation and the function of the coxal glands in ticks. (With 10 Figures in the Text) 172

BATHAM, E. J. Testing arecoline hydrobromide as an anthelminthic for hydatid worms in dogs. (With 4 Figures in the Text) 185

SPRENT, J. F. A. Studies on the life-history of *Bunostomum phlebotomum* (Railliet, 1900), a hookworm parasite of cattle. (With 26 Figures in the Text) 192

SPRENT, J. F. A. Some observations on the bionomics of *Bunostomum phlebotomum*, a hookworm of cattle. (With 2 Figures in the Text) 202

INDEX OF AUTHORS 211

INDEX OF SUBJECTS 212

PAGE
126
131
135
138
152
154
163
172
185
192
202
211
212

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Vol. 37, Nos. 1, 2 January 1946

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THE WATER BALANCE IN *IXODES RICINUS* L. AND CERTAIN OTHER SPECIES OF TICKS

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(With 8 Figures in the Text)

INTRODUCTION

In Britain the sheep tick, *Ixodes ricinus*, is restricted to rough grazings and is absent from good lowland pastures. In heavily tick-infested localities the ground is usually poorly drained, heather, bracken or coarse grasses representing the dominant types of vegetation. Often, decomposing plant material collects to form a 'mat', retentive of moisture, at the base of the growing vegetation (Milne, 1944). Such a habitat not only provides shelter for the developing or inactive stages, but also a microclimate in which the humidity remains high throughout the year. Only a small fraction of the life cycle is actually spent on the hosts; for the remaining period the tick is on the ground.

The various stages of *I. ricinus* are known to display little resistance to desiccation. This is true of the egg (MacLeod, 1935*b*), and of the unfed nymph and larva (MacLeod, 1935*a*). Moreover, the optimal humidity for larval and nymphal development over a wide range of temperatures is saturated air, development failing to proceed below a relative humidity of about 80% (MacLeod, 1934). Reviewing the factors influencing distribution, MacLeod (1936) stresses the importance of humid conditions in permitting survival. Recent ecological studies (Milne, 1944) have laid further emphasis on the correlation between tick distribution and type of vegetation: this relationship is probably indirect; the type of substratum influences the humidity of the microclimate and this in turn may limit distribution.

The present paper is concerned with the manner in which the tick gains or loses water and with the physiology of these exchanges. From a knowledge of the water relations under experimental conditions, and of the conditions of moisture and humidity normally encountered by the sheep tick (Milne, 1944, 1945), we can perceive something of the significance of these under natural conditions.

In general, the terrestrial arthropod may gain water by (*a*) uptake from humid air (see, for example, Mellanby, 1932), (*b*) the absorption of water in contact with the cuticle, (*c*) drinking, (*d*) the absorption of water ingested with the food, and sometimes by (*e*) the utilization of metabolic water. The loss of water may take place by (*f*) evaporation through the cuticle and spiracles, and by (*g*) defaecation and excretion.

Humid air will be shown to form the chief source of water for the unfed tick, although water in contact with the cuticle is also a possible source under experimental conditions. *Ixodes* does not drink. Metabolic water is unlikely to be of any significance, for the fasting tick survives for only a very limited period at low humidities, while in humid air other, and more efficient, mechanisms for gaining water come into operation. Both unfed and engorged stages lose water rapidly by evaporation. In addition, some water is lost in the faeces and excrement which the tick voids after eclosion from the egg, after moulting, and immediately after dropping replete from the host. Whilst it is attached the tick is, of course, gaining water from the ingested blood.

In the following account it will be convenient to consider together the regulation of the water balance in the unfed and in the fully engorged tick (unfed females and engorged nymphs have generally been used) and to treat the water relations of the engorging tick separately. Studies of the resistance of insects to desiccation have often been handicapped by the absence of standards with which to compare the results. In order, therefore, to give added significance to the results obtained with *I. ricinus*, a comparative study of several species of Ixodidae has been made.

METHODS AND MATERIAL

Changes in water content were determined by weighing, and care was exercised in selecting for this purpose ticks which had ceased to void faeces or excrement. Nymphs and unfed adults were weighed on a torsion balance recording to 0·02 mg. Some idea of the relative sizes of the stages used will be conveyed by the statement that the engorged nymph weighs approximately 4 mg., the unfed female and male 1·8 and 1·2 mg. respectively, and the engorged female about 250 mg.

In desiccation experiments ticks were confined individually in cages of metal gauze which were suspended inside 3 × 1 in. specimen tubes containing about 1 in. of the controlling solution. Humidities

were controlled by means of sulphuric acid-water mixtures (Wilson, 1921). All the cultures were kept in approximately saturated air; they were housed in open-ended tubes, plugged with cotton-wool, which were packed vertically into jars with moistened plaster of Paris floors.

The age (from the time of emergence) of the unfed ticks used in these experiments was between 1 and 4 months, save in certain cases where, as specifically stated, the age was more accurately known. The importance of this factor is probably greater than was at first realized.

Stocks of *I. ricinus* were reared in the laboratory, the immature stages usually on hedgehogs and the adults on sheep: these were occasionally supplemented by collections of engorged nymphs from the field. Other species examined have included: Argasidae: *Ornithodorus moubata*; Ixodidae: *Ixodes canisuga*,* *I. hexagonus*,* *Dermacentor andersoni*, *D. reticulatus*,* *Amblyomma cajennense*, *A. maculatum* and *Rhipicephalus sanguineus*.

THE WATER BALANCE IN *IXODES RICINUS*

(i) *The loss of water by evaporation*

The ability to withstand desiccation, and the effect of humidity on the rate of evaporation of water, has been examined by exposing unfed females and males and engorged nymphs to various humidities at 25° C. and weighing them daily. All the ticks were previously maintained in saturated air in the culture tubes. Some curves showing the rate of water loss in unfed females and engorged nymphs are given in Fig. 1; these have been chosen from a number of such curves and serve to illustrate the variability of the material. The results obtained with unfed females may be given in the form of the following summary; five ticks were used at each humidity.

0% R.H. (23·8 mm. S.D.). Unfed females lose up to 50% of their original weight per diem and often fail to survive for 24 hr.

30% R.H. (16·7 mm. S.D.). Females lose up to 20% of their original weight per diem and survive 2–3 days.

50% R.H. (11·9 mm. S.D.). The ticks lose about 10% of their original weight per diem and survive 3–5 days.

70% R.H. (7·1 mm. S.D.). Females lose about 5% of their original weight per diem and survive 4–8 days.

90% R.H. (2·4 mm. S.D.). All females diminish in weight during the first day or so. A few then show slight increases in weight, sometimes eventually attaining a steady state when water is neither gained nor lost (Fig. 1); these ticks may then survive for a considerable time, sometimes for as much as 2 or 3 months. Others, after showing some slight gains, lose water slowly and finally succumb after perhaps 1–2 weeks.

95% R.H. (1·2 mm. S.D.). As at 90% R.H., nearly every female loses some water immediately after introduction into this humidity (Fig. 1), but the rate of water loss falls off within a day or so. These ticks then either increase again in weight, finally reaching a state of equilibrium, or remain at a constant though slightly diminished weight. Nearly every unfed tick develops the power to retain water at this humidity and survives for a considerable period, usually 3 months or more.

The following points emerge from these observations: (i) The rate of water loss, which in turn limits the duration of survival at this temperature, is not related in any simple way to the saturation deficiency of the air. As the humidity is raised the tick loses less water relative to saturation deficiency. At high relative humidities (95% R.H. and over), survival is not related to saturation deficiency at all, for in many cases no overall water loss is sustained over a period of weeks or even months. (ii) Considerable variability as regards water loss and survival is shown at the higher humidities—at 90% R.H. for example. This can be traced to the ability of some individuals to prevent evaporation completely, while others, which are unable to do so, slowly become desiccated. This gives rise to large differences in survival times. (iii) Inspection of the water-loss curves of single individuals shows that the rate of evaporation rarely remains constant. This variability is again most noticeable at high humidities; at 90% R.H., for example, the weight may fluctuate from day to day, sometimes showing small gains and sometimes small losses. Even at 50% R.H., a humidity at which evaporation cannot be prevented for more than a very limited period, the rate of loss may be quite irregular (Fig. 1); typically, evaporation falls off a day or so after introduction into this humidity but increases again before death. This suggests that the tick exercises some control over water loss but that this ability is impaired if the tick is injured through excessive desiccation.

The resistance of males to desiccation is much the same as that of unfed females. At 95% R.H. the male also retains water and survives for a considerable period.

The behaviour of engorged nymphs shows certain contrasting features (Fig. 1). At 0, 30 and 50% R.H. water loss is rapid, the amount evaporated per diem being greater than in unfed females; this is possibly due to their larger effective surface area. But at 90 and 95% R.H. the engorged nymphs still evaporate water slowly and eventually dry up.

These findings agree fairly closely with those of

* Species occurring in Britain.

MacLeod (1935*a*), who worked with large batches of unfed larvae and nymphs. MacLeod notes that at low humidities survival can be approximately correlated with saturation deficiency, but that at higher humidities the correlation becomes less exact this. MacLeod also suggests that this breakdown of the water-conserving mechanism in the nymph is due to the failure to close the spiracles. Other results, which are discussed below, point to a different explanation.

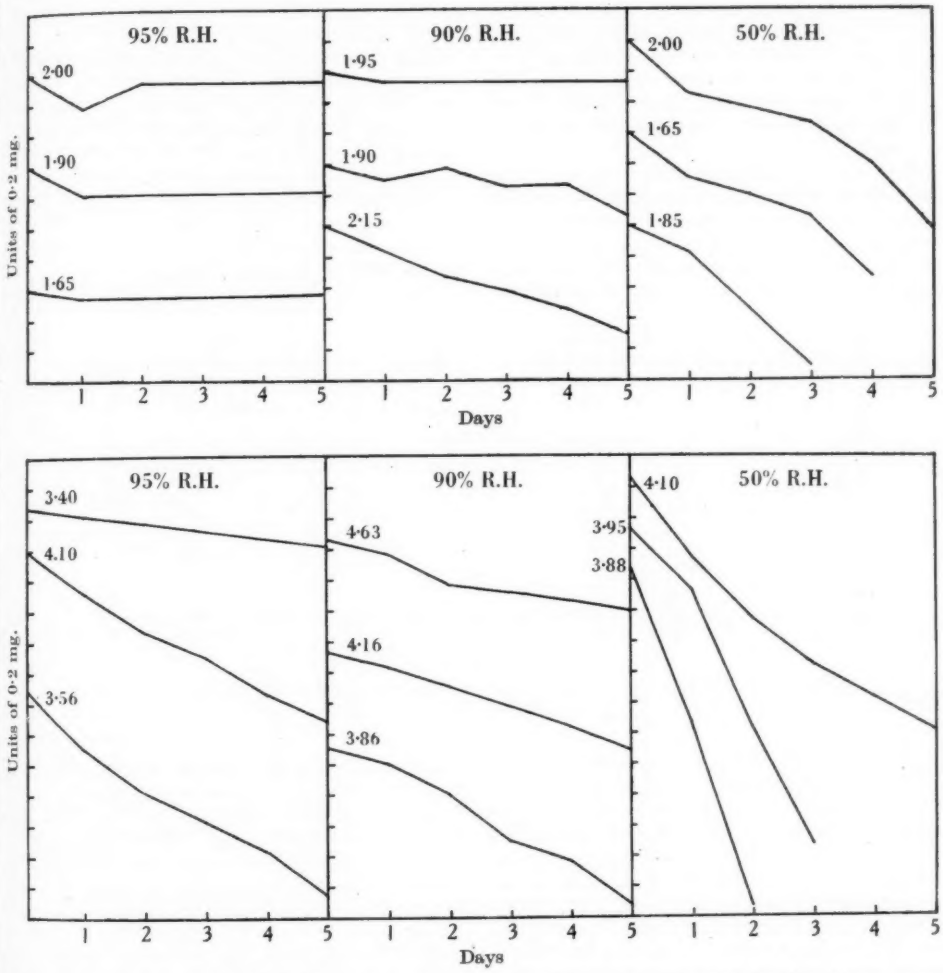

Fig. 1. Rate of water loss from *Ixodes ricinus* at various humidities. Above, unfed females; below, engorged nymphs. As in other figures three ticks are shown at each humidity. The initial weight is given near the origin of each curve.

until, as saturation is approached, survival is more closely related to relative humidity. He also finds that, as compared with water loss at higher humidities, nymphs lose more water in dry air than is to be expected if loss is determined solely by saturation deficiency. The present results offer confirmation of

(ii) *Water uptake at high humidities*

The slight increases in weight noted among unfed females exposed to 90 % and, more particularly, to 95 % R.H., suggested that the desiccated tick might be able to take up water from nearly saturated air.

Among insects, the mealworm *Tenebrio molitor* (Mellanby, 1932) and the grasshopper *Chortophaga viridifasciata* (Ludwig, 1937) are known to possess this ability. In order to investigate this further, unfed females and males, engorged nymphs and engorged females were desiccated at 50% R.H., 25°C., for 2 days and were then transferred to 90, 95 or 100% R.H. The changes in weight shown by certain unfed females are recorded in Fig. 2.

At 90% R.H. some unfed females are able to gain water slowly after desiccation; others, however, lose water. The weight may sometimes fluctuate from day to day.

At 95% R.H. the desiccated females take up water initially at about the same rate as they lose it at 70% R.H., that is, amounting to some 0·15 mg. or 5–10% of their original weight per diem. After 2–3 days at 95% R.H. most have returned to their original weight which is then maintained without change. Frequently, though not invariably, the final weight attained closely approximates to the weight before desiccation. It is clear that the water evaporated is eventually removed from, and water taken up, returned to, the haemolymph, for the tick, visibly shrunken after desiccation, fills out as water is gained. It should also be added that this humidity is sufficiently low to exclude the possibility that uptake could be due to the absorption of condensed moisture from the surface of the cuticle.

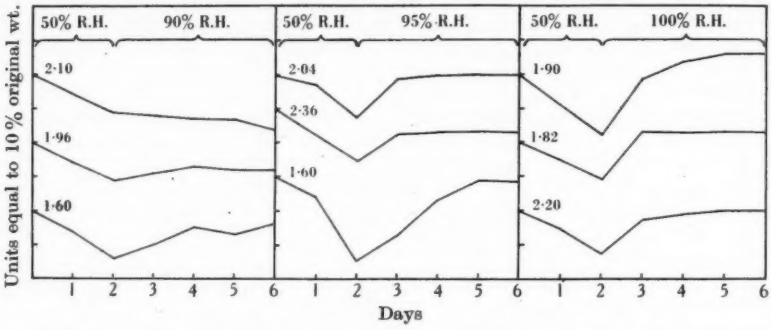

Fig. 2. Uptake of water at high humidities by unfed females after desiccation for 2 days at 50% R.H.

In saturated air the rate of uptake of water is very rapid (Fig. 2) and may be equal to as much as 0·3 mg. per diem, equivalent to 10–20% of the original weight. The rate of gain of water in saturated air is thus approximately the same as the rate of evaporation at 30% R.H. As is the case at 95% R.H., the rate of increase in weight diminishes as the original weight is regained, the weight being finally maintained constant. Often, however, uptake is exaggerated and rather more water is gained than has been lost during desiccation (Fig. 2). Regulation in this instance is less perfect.

The loss of water by evaporation and its replacement in humid air is an event which can take place repeatedly. Thus, when ticks were exposed on alternate days to humidities of 70 and 95% R.H., the fluctuations in weight often showed great regularity over a period of 2 weeks or more.

The results described in this and in the preceding section show that the unfed tick usually reaches a state of equilibrium when the humidity lies within the range 90–95% R.H. At relative humidities above the point of equilibrium, the tick gains water, while at lower humidities it loses water.

The equilibrium humidities in ten unfed females (of varying ages) were next determined. The ticks were desiccated and then exposed to successively higher humidities until evaporation ceased and uptake became just perceptible. The humidities at which equilibrium was attained were found to range from 86 to 96% R.H. (mean = 92% R.H.).*

Males, like unfed females, take up water from 95% R.H. after desiccation. In contrast, engorged nymphs usually fail to do so, but since they are unable to prevent evaporation at 95% (p. 2) this is to be expected. The ticks examined have included overwintering nymphs (engorged in September and used for experiment the following March) and nymphs fed only 2 weeks previously; the ticks in the two batches were all active and none showed signs of an impending moult. Of eight nymphs

* Unfed females, after desiccation, may sometimes fail to take up water even from saturated air. This is usually true of newly emerged ticks which have not yet discharged the residue from the gut, and often of old individuals which have remained in a fasting condition for many months. There is some reason to believe that as the tick ages, the ability to take up water, and also to withstand desiccation, declines.

tested at 95% R.H. after the initial desiccation, only one, an overwintering specimen, was found to gain water, the rate of uptake being about 1% of the original weight per diem; the remainder continued to evaporate water at a diminished rate. In saturated air recently engorged nymphs, after desiccation, remained at a constant weight for several weeks. Five fully engorged females tested in a similar manner also proved to lack the ability of taking up water from 95% R.H. or from saturated air. Six females were then allowed to attach to sheep, and were removed in pairs, 2, 4 and 6 days after attachment. The partially engorged ticks were desiccated as previously and transferred to 95% R.H. Again, none were able to gain water but instead continued to lose water slowly. Evidently the power of regulating evaporation, and of taking up water at high humidities, is lost soon after the tick begins to feed.

Table 1. *Weights of unfed females, expressed as percentages of the original weight, after desiccation at 50% R.H., 25°C., for 1 day, and after subsequent exposure for 2 days to the saturation deficiencies shown. Five ticks were tested at each saturation deficiency*

25°C.						20·5°C.					
100% R.H. S.D. 0 mm.		95% R.H. S.D. 1·2 mm.		90% R.H. S.D. 2·4 mm.		100% R.H. S.D. 0 mm.		95% R.H. S.D. 0·9 mm.		90% R.H. S.D. 1·8 mm.	
94	99	97	100	82	71	93	100	94	100	90	86
91	100	94	94	90	100	90	106	92	100	88	65
90	100	97	100	89	85	77	100	97	100	90	93
91	94	94	100	85	80	96	102	93	99	74	62
91	100	94	100	85	82	94	100	85	86	88	69
15°C.						9°C.					
100% R.H. S.D. 0 mm.		95% R.H. S.D. 0·64 mm.		90% R.H. S.D. 1·3 mm.		100% R.H. S.D. 0 mm.		95% R.H. S.D. 0·43 mm.		90% R.H. S.D. 0·86 mm.	
95	100	96	101	95	98	87	94	87	88	90	77
92	100	90	90	93	96	92	99	90	94	93	91
91	101	87	96	94	86	90	94	93	93	88	81
86	99	88	86	92	92	90	98	93	97	90	86
90	102	93	98	94	90	83	100	91	91	80	70

(iii) *The uptake and retention of water at various temperatures*

We have seen that at 25°C. the unfed tick retains water when exposed to high humidities and takes up water after desiccation. Further experiments were undertaken in order to determine whether retention and uptake near the point of equilibrium is governed by the relative humidity or saturation deficiency of the air, and to determine also the temperature range over which uptake is possible.

Unfed females, after an initial desiccation for 24 hr. at 50% R.H., 25°C., were exposed singly for 2 days to humidities of 100, 95 and 90% R.H., at temperatures of 25, 20·5, 15 and 9°C. Five ticks were kept at each saturation deficiency. The results are given in Table 1. Over the temperature range 25–15°C. the capacity for taking up water appears to be much the same. In saturated air 15 out of 15 ticks gained water and at 95% R.H., 11 out of 15; at 90% R.H. 13 out of 15 lost water while two showed overall gains. At 9°C., however, the rate of uptake appears to be less rapid, for at 95% R.H., although no water was lost, comparatively little was gained after 2 days' exposure to this humidity; in saturated air also only one out of five returned to the initial weight, compared with four out of five at 25°C. At 90% R.H., 9°C. unfed females lost water at approximately the same rate as at higher temperatures.

Two conclusions can be drawn from these results: (i) If the humidity is near the point of equilibrium the exchanges of water are determined by the relative humidity; for example, at 25°C., 95% R.H. (S.D. 1·2 mm. Hg), nearly all ticks take up water, yet at 15°C., 90% R.H. (S.D. 1·3 mm. Hg), nearly all lose water. Similarly, although the power to retain and to take water slowly still persists at this temperature, all the ticks lost water at 9°C., 90% R.H. (S.D. 0·9 mm. Hg). (ii) At low temperatures the rate of uptake appears to diminish slightly; this is to be expected if the uptake of water is an active process.

Further evidence on the ability of ticks to retain water at high humidities has been obtained by removing unfed females from saturated air and exposing them for successive 4-day periods to the following temperatures and humidities: 15°C., 95% R.H. (S.D. 0·6 mm. Hg); 34°C., 95% R.H. (S.D. 2·0 mm. Hg); 15°C., 85% R.H. (S.D. 1·9 mm. Hg).

The results are shown in Table 2. After the initial exposure to 95% R.H. most of the ticks diminish slightly in weight, as is to be expected after previous exposure to saturated air. At 34°C., 95% R.H., not only is water retained, but most individuals show slight increases in weight, in spite of the fact that the saturation deficiency is three times as great. On the other hand, when transferred to 15°C., 85% R.H., with a saturation deficiency slightly less, all the ticks lose water and eventually dry up.

The findings of Mellanby (1932) with regard to the uptake of water by *Tenebrio* larvae were very similar. In the mealworm, as in *Ixodes*, the relative humidity of the air determines whether water is lost or gained if the humidity is near the point of equilibrium. The mean value for the equilibrium humidity in *I. ricinus*, 92% R.H., compares with values of about 88% R.H. in *Tenebrio* and 82% R.H. in *Chortophaga* (Ludwig, 1937).

Table 2. *The retention of water by unfed females exposed successively to humidities of* 95, 95 *and* 85% R.H. *at the temperatures shown. Weights expressed as percentages of the original weight*

100% R.H. 25°C. S.D. 0 mm.	95% R.H. 15°C. S.D. 0·64 mm.	95% R.H. 34°C. S.D. 2·0 mm.	85% R.H. 15°C. S.D. 1·9 mm.
100	96	99	84
100	98	99	76
100	96	96	79
100	97	99	60
100	96	101	70

(iv) *Uptake from water in contact with the cuticle*

It will be shown in § (v) that the exchange of water in *Ixodes* takes place mainly through the cuticle. Additional information concerning the passage of water through the cuticle and its regulation has been obtained by studying the effect of water in contact with the cuticle. To carry this out, unfed females and engorged nymphs were held between pads of wet filter paper and were weighed daily after careful drying. Unfed ticks trapped in this manner do not usually become asphyxiated as they do when submerged under water.

Fig. 3*a* records the changes in weight of three females from saturated air when held in contact with water, and Fig. 3*b* those of other ticks which received an initial desiccation (1 day at 50% R.H., 25°C.). The difference in behaviour is often striking. While the normal ticks absorb water only very slowly, the desiccated ticks take up water rapidly and usually continue to do so after the normal water content has been restored; these latter soon become abnormally distended with fluid and, when liberated, their movements are seen to become gradually more sluggish as their state of distension increases. An increase in weight of 25% is usually fatal, but smaller increases can usually be tolerated for at least a limited period.

These results suggest that the normal unfed tick is to some extent able to prevent the entry of water in contact with the cuticle; after desiccation, however, water is taken up so rapidly from this source that uptake is exaggerated and the ability to control its entry is finally lost.

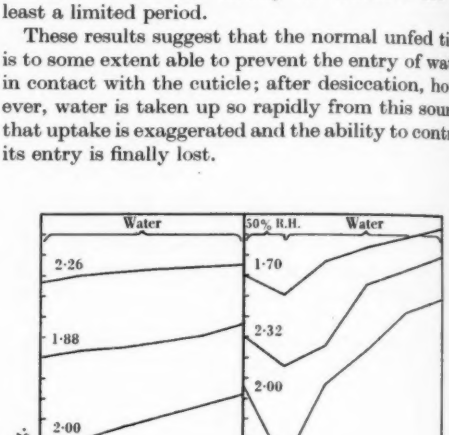

Fig. 3. Uptake by normal and desiccated ticks from water in contact with the cuticle. *a–b*, unfed females; *c–d*, engorged nymphs. In *a* and *c* the ticks had previously been exposed to saturated air; in *b* and *d* they received an initial desiccation at 50% R.H. of 1 and 2 days respectively.

The behaviour of engorged nymphs is more variable (Fig. 3 *c*, *d*). Normal nymphs, after previous exposure to saturated air, usually remain at a constant weight for several days when placed in contact with water, but then begin to increase in weight (Fig. 3*c*). Some desiccated nymphs take up water immediately while others fail to do so for several days (Fig. 3*d*). The engorged nymph also appears, therefore, to be able to prevent the entry of water for a limited period, but the difference in the rate of uptake by the normal and desiccated nymphs is much less marked than in unfed females.

(v) *The site of water exchange*

We have seen that the unfed tick loses water by evaporation (*a*), and gains water by uptake from high humidities (*b*) or by absorption from liquid water (*c*). The exchanges (*a*) and (*b*) might conceivably take place through the cuticle or through the spiracles, while (*c*) might be due to absorption through the cuticle or to drinking. The spiracles and respiratory system in *Ixodes ricinus* have been described by Falke (1931); spiracles are absent in the larva, while nymph and adult possess a single pair. Descriptions of the cuticle are to be found in Bonnet (1907), Nordenskiöld (1908) and Ruser (1933). An account of certain of its features is given in § (vi). Here we may merely note that the cuticle is everywhere continuous save where it is penetrated by the ducts of the dermal glands (see below); these are so numerous that account has to be taken of them when considering the site of water exchange.

The site of evaporation

Unfed females were exposed to humidities of 90, 85, 70 and 50 % R.H.; after 1 or 2 days their spiracles were covered with cellulose paint and they were returned to the same humidity. Fig. 4, which records the daily water loss expressed as a percentage of the original weight, shows that in several instances more water is evaporated when the spiracles are covered than when they are free. This is particularly noticeable at 70 and 50 % R.H. and reflects the increase in the rate of evaporation occurring, as noted above, when the tick is injured through excessive desiccation. This water must be lost through the cuticle; the increased rate of loss clearly cannot be due to failure to close the spiracles.

Evaporation through the cuticle can also be demonstrated by painting the dorsum with a solution of anhydrous cobalt chloride in celloidin (Eder, 1940), allowing the film to dry and then placing the tick in dry air for 6 hr. When the membrane is peeled off it has assumed a rose pink colour.

The effect of CO_2 on the rate of evaporation at various humidities was also investigated. It is well known that in many insects (Wigglesworth, 1939), and also in the tick *Ornithodorus moubata* (Mellanby, 1935), the rate of water loss is increased several-fold when the spiracles are caused to remain open by exposing the arthropod to CO_2. According to Falke (1931) the spiracles of *Ixodes ricinus* are provided with a closing mechanism.

As in the previous experiment unfed females were kept at humidities of 90, 85, 70 and 50 % R.H. and were weighed daily; after exposure for 1 or 2 days to these humidities 5 % CO_2 was introduced into the desiccator for 1 day. Fig. 4 shows that at a given humidity water loss in the presence of CO_2 may be either greater or less than in CO_2-free air. We have therefore no certain means of knowing whether the spiracles are caused to open by CO_2, but, assuming this to be so, the results may be interpreted in the following way. At low humidities any extra water loss incurred by the opening of the spiracles may be entirely masked by the grosser changes taking place in the rate of evaporation from the cuticle. At higher humidities, and in particular near the equilibrium humidity, when little or no water is lost through the cuticle, the effects of increased evaporation through the spiracles might be expected to be more conspicuous; this may perhaps account for the high rate of water loss shown by the tick exposed to CO_2 at 90 % R.H. (Fig. 4); on the other hand, no such effect is noticeable in the individual desiccated at 85 % R.H., and it is not improbable that near the equilibrium humidity any small losses from the spiracles are made good by the uptake of water through the cuticle (see below).

One may conclude from these results that in *I. ricinus* water loss takes place almost exclusively through the cuticle. The presence of the numerous dermal ducts may exert some effect on evaporation, but that the bulk of the water is evaporated from the surface of the epicuticle appears likely for the following reason. At 25° C. and 50 % R.H. the unfed female loses about 0·20 mg. of water per diem, the engorged female as much as 10 mg. per diem. As the tick fills with blood the apparent surface area increases some twenty times, but the number of ducts remains unaltered. It follows that in the engorged tick at least, the greater part of the water evaporated is certainly lost from the epicuticle.

The site of water uptake

It has been suggested by Mellanby (1932) that the ability of mealworms to take up water at high humidities is associated with the secretory activity of the tracheolar endings. This has been tested in *Ixodes* by desiccating unfed females, covering the spiracles and exposing them to 95 % R.H. A comparison of the results (Fig. 5) with those given in Fig. 2 shows clearly that blocking the spiracles in no way impairs the ability of the tick to take up water at this humidity. Uptake must then be occurring over the surface of the cuticle in general. Fortunately, nearly all ticks survive in an active state for a number of days with the spiracles covered, but occasionally individuals succumb rapidly to asphyxia and will then never take up water either from 95 % R.H. or from higher humidities. It is interesting that such asphyxiated ticks usually diminish in weight at 95 % R.H. and thus evidently fail also to retain water. If the spiracle blocks are removed these ticks sometimes recover and regain their powers of retention or uptake of water at this humidity. Such individual variations in the ability to survive with the spiracles covered are doubtless

due to the varying efficiency of cutaneous respiration.

Absorption can also take place from liquid water in contact with the cuticle, for the water uptake of ticks held between wet filter paper is unaffected by covering the spiracles or by painting over the proboscis. *Ixodes* does not drink water and is never seen probing moist objects. An attempt was made to demonstrate the entry of water through the cuticle by immersing ticks in saturated solutions of various dyes (neutral red, brilliant green, crystal violet and toluidine blue); the dorsal cuticle was afterwards dissected off and mounted whole. The results were similar irrespective of the dye used; in

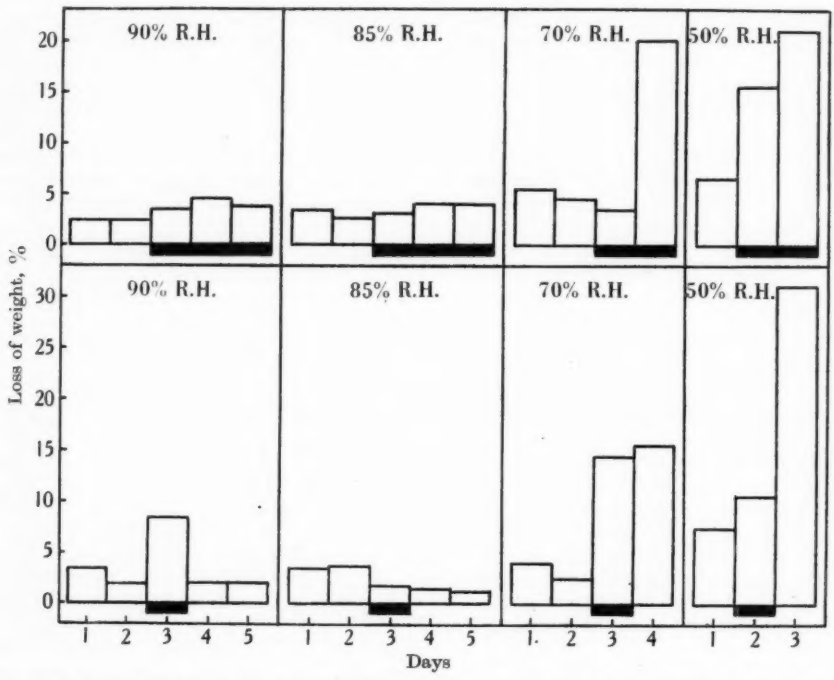

Fig. 4. Effect of covering the spiracles (above) and of exposure to 5% CO_2 (below) on the daily water loss from unfed females maintained at various constant humidities. Periods when these treatments were applied are shown by bars.

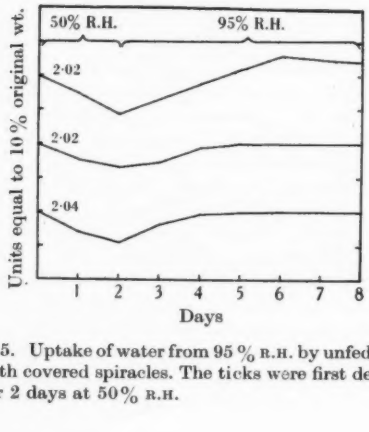

Fig. 5. Uptake of water from 95% R.H. by unfed females with covered spiracles. The ticks were first desiccated for 2 days at 50% R.H.

no case was the dye found to penetrate the epicuticle, but minute deposits of solid dye were sometimes found in the lumen of the dermal ducts; their size seemed to indicate that the bulk of the absorption was taking place elsewhere.

(vi) *The structure of the cuticle and epidermis*

Since, as has been shown, water exchange in *Ixodes* takes place mainly through the cuticle, a brief description of cuticle and epidermis in the unfed tick may be given at this juncture.

The cuticle and dermal glands

Two principal layers can be distinguished in the integument, and endocuticle and epicuticle (Fig. 6). In the female the endocuticle of the legs, capitulum

and scutum is rigid, pigmented and highly sclerotized, while that of the alloscutum,* destined to become enormously expanded in the replete female, is soft, colourless and extensible; these two types of cuticle merge into one another at the junction of scutum and alloscutum. The outer layer, or epicuticle, is a well-defined membrane, little more than 1μ in thickness. Over the scutum, legs, etc., the epicuticle is flat and difficult to detect, but over the alloscutum, where it is thrown into deep folds, its characteristic colour, a deep orange, renders it easily distinguishable in sections from the colourless endocuticle.

In the male almost the entire body surface is hard and pigmented and only small areas of unsclerotized endocuticle are left between the plates of the conscutum. In consequence, the male can only take a minute blood meal; often it does not feed at all.

properties of the cast skins, for whereas the nymphal exuvium of *I. ricinus* is a rigid structure, retaining the shape of the nymph, that of *I. canisuga* or *I. hexagonus* is exceedingly tenuous and immediately crumples up after being shed. Pryor has pointed out in this connexion that the introduction of aromatic cross-linkages into the protein framework of the cuticle greatly increases the mechanical rigidity.

It is unlikely, however, that the epicuticle in the unfed tick consists of a simple protein layer, for its surface possesses obvious hydrofuge properties; possibly the protein layer is impregnated or perhaps overlaid by a lipoid. If this view, which is in harmony with recent ideas of the nature of the insect epicuticle (Wigglesworth, 1944), is correct, this layer will doubtless have an important waterproofing function. In this connexion the appearance of the cuticle in any of the living engorged stages of *I. ricinus* is suggestive. The epicuticle of the recently

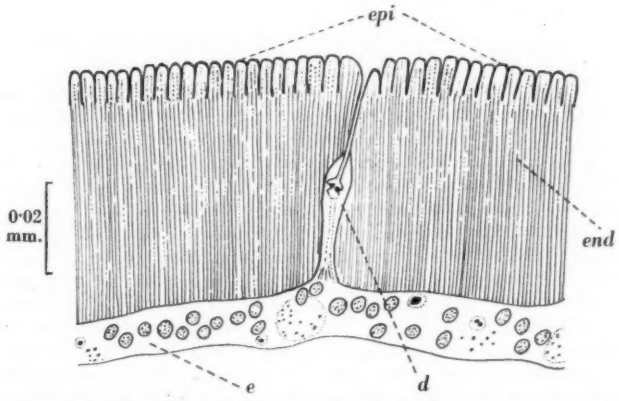

Fig. 6. Transverse section of the cuticle and epidermis of the unfed female of *Ixodes ricinus*. *e*, epidermis; *end*, endocuticle and pore canals; *epi*, epicuticle; *d*, duct of dermal gland.

Cast nymphal skins, consisting only of epicuticle, give a positive reaction with Millon's reagent and are probably mainly protein in nature. By analogy with insects (Pryor, 1940) it appears likely that the darkening of the epicuticle (and of some regions of the endocuticle) which takes place after moulting, is due to a tanning process. The alloscutal epicuticle of other species of Ixodidae differ considerably in colour. In *Rhipicephalus sanguineus*, *Amblyomma maculatum* and *Dermacentor andersoni*, for example, the epicuticle is brown or chocolate-coloured, while in *Ixodes canisuga* and *I. hexagonus* it is colourless and is presumably untanned. This latter conclusion is also supported by observations on the mechanical

engorged female, for example, has a shiny, glistening appearance, and if the tick is left in saturated air for a few days, examination with the binocular reveals the presence of little pools of oily secretion exuding from the ducts of the dermal glands and gradually spreading over the surface of the cuticle. This process has been figured by Falke (1931).

The cuticle is everywhere traversed by the ducts of the dermal glands: these have a complicated structure (Fig. 6) and are of the same colour as the epicuticle with which they are continuous. To the exterior the ducts open freely on to the surface of the cuticle, while at the base of the endocuticle their open ends lie against epidermal cells. In this latter respect they differ from the dermal glands of *Rhodnius* (Wigglesworth, 1933), which are entirely lined by a delicate pellicle of cuticle. In the unfed female the 'gland' cells at the base of the ducts are

* In the Ixodidae the region posterior to the scutum in the female has been termed the 'alloscutum' (Schulze, 1930); in the male, scutum + alloscutum are referred to as the 'conscutum'.

indistinguishable from other epidermal cells, but become enormously hypertrophied when the tick engorges; their function is then to secrete a continuation of the original duct (Nordenskiöld, 1908) and also to elaborate or pass on the oily secretion appearing at the surface of the cuticle (see above). In the unfed female there are about 1000 ducts per sq.mm. over the alloscutum and about 400 per sq.mm. over the scutum. The total number of ducts in a single tick will be of the order of 3000–7000.

The pore canals and epidermal cells

In frozen sections or even in balsam preparations the pore canals are readily distinguishable as fine striae extending through the endocuticle from epidermis to epicuticle (Fig. 6). Their course is effectively demonstrated by the use of the rapid Golgi technique (Ruser, 1933). In the unfed female the canals are arranged in bundles: beneath the epicuticle, which they never penetrate, the canals anastomose and send out short branches which end blindly. It is stated by Nordenskiöld (1908) that each bundle of pore canals runs proximally into a single epidermal cell.

There is evidence that in certain insects, the contents of the pore canals becomes chitinized and finally converted into solid rod-like objects during the later stages of deposition of the cuticle (Dennell, 1943). This is certainly not the case in the unfed tick for, as in *Rhodnius* (Wigglesworth, 1933), the pore canals may become filled with air when detached pieces of cuticle are dried. Probably, therefore, the pore canals in *Ixodes* are to be regarded as discrete channels occupied by filaments of cytoplasm extending from the body of the epidermal cell. Even in insects such as *Sarcophaga* in which they are particularly large, the pore canals number about 60 per epidermal cell (Dennell, 1943), while in the cockroach they are estimated as numbering about 200 per cell (Richards & Anderson, 1942). Quite clearly the effective surface of the cell will be enormously increased. As pointed out by Wigglesworth (1933), this arrangement also permits the epidermal cells to exert their influence directly, by the passage of substances synthesized in the cell body, on either endocuticle, or, since the tips of the pore canals are in such close proximity, on epicuticle also. But besides their probable importance in conveying synthesized materials to the cuticle, it is legitimate to suppose that the pore canals play an important role in the exchange of water between haemocoele and the exterior. This question has been discussed by Richards & Anderson (1942), who believe, however, that the pore canals in the cockroach are normally occupied by fluid, perhaps a salt solution, and not by cytoplasm. In *Ixodes* it seems more probable that the canals do in fact contain cytoplasm, for there is reason to believe that as the tick engorges, new cuticular material, synthesized by the epidermal cells, passes up the pore canals.

It is clear that water evaporated from the epicuticle is replaced by water withdrawn by the epidermal cells from the haemocoele. When water is transferred in the opposite direction at high humidities, the epidermal cells and pore canals, as seems reasonable from their nature and arrangement, can perhaps be pictured as actively taking up water from the epicuticle and endocuticle and secreting it into the haemocoele. The large surface areas exposed by the branched and tufted extremities of the pore canals may be of significance in this respect.

(vii) *The regulation of the water balance*

Having considered the sites of water exchange, some further points connected with the regulation of the balance may now be examined.

The regulation of water uptake

After desiccation the unfed tick continues to gain water when exposed to a high humidity only so long as the water content is depleted, further increases in weight ceasing when the normal balance has been restored. Since, as we have seen, the respiratory system plays no direct part in water uptake, this cannot be due to the closure of the spiracles. Two alternative explanations appear possible. First, the tick may continue to take up water through the cuticle, even when the water content is normal, but may maintain the balance by excreting the excess. Or secondly, the epidermal cells may cease to secrete water when sufficient has been absorbed.

These possibilities have been tested by desiccating unfed females, occluding the anus with cellulose paint (to prevent excretion of water by the Malpighian tubes) and exposing them to 95% R.H. Under these conditions it was found that uptake does in fact still cease when the normal water content has been restored. It is clear then that a progressive decline in secretory activity must take place.

In the light of these results it was interesting to see whether ticks which had absorbed excess water through the cuticle could restore the normal balance by excretion. Unfed females and engorged nymphs which, after an initial desiccation, were held in contact with water for varying lengths of time, were finally removed to saturated air. These ticks were then divided into two groups; in one of these, excretion by the Malpighian tubes was prevented by blocking the anus; the other group, in which the anus was left free, served as a control.

Fig. 7 shows clearly that both unfed females and engorged nymphs are able to eliminate excess water and that this takes place even when the Malpighian

tubes are out of action. Such activity must also then be ascribed to the epidermal cells.

An attempt was made to observe this elimination of water taking place. Engorged nymphs which had absorbed excess water were placed in a small chamber on a microscope stage and were observed periodically under the low power. The humidity in the chamber was maintained at about 86% R.H. Droplets of water, which were not due to condensation, could be seen forming at the surface of the cuticle, but these never became very large, presumably evaporating rapidly into the humid air. It was not possible to decide whether the droplets were derived from the dermal glands or from the general surface of the epicuticle.

If the surface of the tick were maintained at a temperature slightly above that of the surrounding air, some evaporation into 'saturated' air might conceivably take place. But since the tick with a normal water content does not so evaporate water—the weight is in fact maintained constant in saturated air—this result seems to imply that the loss of fluid is an active process.

Fig. 7 also shows that regulation may be very imperfect, the tick often failing to return to the original weight when replaced in saturated air. Both unfed females and engorged nymphs show this. After exposure to water for some days the loss of fluid may continue long after the normal balance has been restored and in extreme cases the tick may dry up. This takes place in spite of the fact that the ticks are usually active and apparently unharmed when removed from contact with water.

The retention of water

The observations described in § (i) show that the water loss from the unfed female increases when desiccation becomes excessive. As the same effect is also found, just as strikingly, when the spiracles are covered (Fig. 4), it appears that the rate of loss through the cuticle must increase considerably after injury to the epidermal cells. These experiments have been repeated in the following way. Unfed females were kept at humidities of 100, 95, 70, 50 and 30% R.H. for 1 day, killed by exposure to ammonia vapour for 30 min. and then returned for 1 day to the same humidity. Save during the short exposure to ammonia, all the external openings, including spiracles, anus and proboscis, were covered by painting them over with cellulose lacquer. The ticks in this experiment were previously kept at 95% R.H.

The daily losses of water before and after death, expressed as percentages of the original weight, were as follows: at 100% R.H., 0 and 15%; at 95% R.H., 0 and 25%; at 70% R.H., 5 and 36%; at 50% R.H., 12 and 38%; and at 30% R.H., 28 and 32%. Evidently the ability to retain water at humidities above the point of equilibrium (e.g. at 95% R.H.) is entirely lost at death. As the humidity is lowered the rate of evaporation from the cuticle of the living tick becomes more nearly equal to the post-mortem rate of loss; in this respect, exposure to a low humidity, in leading to a rapid breakdown in the water-conserving mechanism, simulates the action of any lethal agent. Since, however, the weight diminishes even in saturated air, it seems likely that some fluid is also lost from the cuticle, perhaps from the ducts of the dermal glands. When taken in conjunction with the observation that the rate of loss from the living tick may increase before irreversible effects are produced, and presumably, therefore, before any loss of fluid takes place, these results may imply also that the rate of evaporation from the epicuticle increases at death or during injury. This would also lead to the inference that

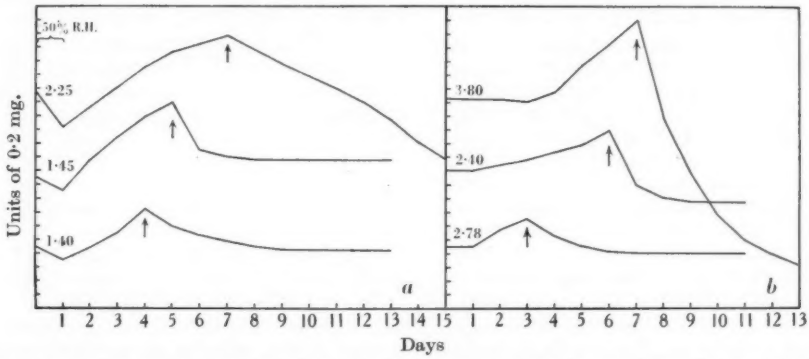

Fig. 7. Loss of excess water through the cuticle in unfed females (*a*) and engorged nymphs (*b*). All the ticks had the anus occluded. At the points marked by arrows the ticks were removed from contact with water and placed in saturated air. The females received an initial desiccation at 50% R.H.

as the tick undergoes desiccation it is actively opposing the loss of water.

This conclusion was also tested by exposing unfed females to 95% R.H.—at which humidity they remained at a constant weight—and then introducing containers with solid potassium cyanide into the desiccators. After 24 hr. about half the ticks showed signs of poisoning and were incapable of walking although still able to cling to objects with their tarsal climbing organs. These affected ticks immediately began to lose water at 95% R.H. and dried up within 4 or 5 days. Although there was no recovery after cyanide treatment, it seems likely that the initial failure to retain water was in this case due to inhibition of the normal respiratory processes.

The active retention of water by the desiccated tick is also suggested by the following experiment. Unfed females, desiccated at 50% R.H., were exposed to successively higher humidities until the equilibrium humidity was reached; they were then left at this humidity for as long as possible and were then transferred to 95% R.H. Some ticks remained at the equilibrium humidity for as long as 2 weeks, neither gaining nor losing water, but when exposed to 95% R.H. immediately began to take up water. This result shows that adaptation does not occur if the water content is depleted and suggests that secretory activity is maintained so long as the tick is in a desiccated condition.

It has been shown (p. 2) that ticks adapted to saturated air are at first unable to retain water, even at 95% R.H., but that the ability to do so appears within a day or so. The question naturally arises as to whether the efficiency of retention is still further increased when the desiccated tick is 'acclimatized' to successively lower humidities. Unfed females, desiccated at 50% R.H. for 1 day, were transferred to 90% R.H. for 2 days; at this humidity they slowly lost water. They were then exposed successively to 85, 70 and 50% R.H., before being finally returned to 90% R.H. At this latter humidity the ticks still continued to lose water at the same rate as previously. One may conclude that once secretory activity is brought into action by exposure to a humidity below the equilibrium the efficiency in retaining water rapidly becomes maximal; certainly the efficiency cannot be increased by more than a few per cent relative humidity.

(viii) *The water relations of the engorging tick*

Whilst attached to the host the tick is gaining water from the ingested blood and is losing water in the faeces and excrement and by evaporation from the cuticle (and to a lesser extent from the spiracles). The following experiments were aimed at determining the quantity of blood taken by the female tick at a meal and the subsequent fate of the ingested water.

The female remains attached to the host for about 8 days. During this period growth in volume is not constant. Indeed two more or less distinct phases can be distinguished, the first occupying about 7 and the second about 1 day. During the first phase the tick increases steadily in weight and also in surface area. Dissections of such partially engorged ticks show that the mid-gut caeca, although larger than in the unfed female, retain their original shape and contain comparatively little solid matter. Digestion and absorption are proceeding actively. At this time also, the epidermal cells become greatly hypertrophied and begin to lay down new cuticle, thereby adding considerably to the thickness of the integument. By the end of this phase the tick, initially weighing about 2 mg., will have attained a weight of, say, 50 mg. At this juncture a large volume of blood is rapidly taken into the gut, the cuticle is stretched and becomes thinner and the haemocoele is temporarily obliterated. The tick, weighing perhaps 250 mg., is now ready to drop from the host.

If a recently engorged female is dissected the gut contents are found to consist of a semi-solid, undigested mass of blood of a cheesy consistency. Evidently a large part of the water has been removed. During the initial phase when cellular activity is at its height, excrement collects in the rectal sac and is periodically discharged as a white pasty mass. Normally, little water appears to be excreted, but occasionally, as the tick detaches itself, a drop of more dilute urine, almost free from guanine, is excreted. At intervals much red, undigested blood is also voided by the engorging tick.

The water balance in the engorging female

To reach a quantitative estimate of the water eliminated we require to know (*a*) the total volume of the ingested blood (from which, knowing the specific gravity and the proportion of water to solids, the total water taken in may be calculated) and (*b*) the water retained in the haemocoele, gut and other tissues. The size of the blood meal cannot be obtained simply by weighing, as part of the meal is digested before engorging is complete. This value has therefore been arrived at indirectly by estimating the ferric iron in the gut and calculating the volume from the known iron content of the host's blood. The procedure was as follows: the engorged tick was weighed and dried over concentrated sulphuric acid at 50° C. until there was no further change in weight. The dried cuticle was then dissected off and the gut contents, after pulverization, were digested in 10 c.c. concentrated sulphuric acid, organic matter being oxidized with 2% potassium

permanganate. The solution was made up to 25 c.c. and aliquot portions were then compared in a Duboscq colorimeter with a standard solution containing 0·01 mg. Fe per c.c. Potassium thiocyanate was used as the indicator (Breuer & Militzer, 1938).

A preliminary analysis of a sample of sheep blood by this method gave a value of 0·50 mg. Fe per c.c. of blood. The water content was 81 %. The specific gravity of the blood was taken as 1·055.

The results of five analyses are shown in Table 3. The first individual may be cited as typical. We see that a female, originally weighing about 2 mg. and after engorging, 252 mg., takes a meal of 0·52 c.c. or 550 mg. of blood; this contains about 450 mg. of water, of which only 137 mg., or about one-third, is retained at the end of engorgement, largely no doubt in the gut. About 313 mg. or two-thirds of the water contained in the meal is therefore eliminated.

Table 3. *The size of the blood meal and the water relations of five engorged females. The difference between water ingested with the meal and that retained in the tissues represents water eliminated during engorgement*

Original weight of tick	Dry weight	Water retained		Blood ingested		Water ingested
mg.	mg.	mg.	%	c.c.	mg.	mg.
252	115	137	51	0·52	550	450
200	98	102	51	0·41	430	350
231	111	120	52	0·53	560	450
265	128	137	52	0·61	640	520
237	109	128	54	0·58	610	490

It would have been interesting to estimate the water lost by evaporation during engorgement by occluding the anus and so preventing the excretion of water by the Malpighian tubes. Unfortnately, no entirely satisfactory means of doing this was found owing to the greasy surface of the cuticle and its rapid growth and expansion. Nevertheless, it seems probable that in *I. ricinus* a substantial, though unknown, fraction of the water eliminated is normally lost by evaporation from the cuticle. This is suggested by a consideration of the permeability of the cuticle at temperatures and humidities to which the engorging tick must normally be exposed. This is discussed below.

The evaporation of water from the engorged female

Fully engorged ticks with the spiracles covered were desiccated at humidities of 0 and 50 % R.H., and at temperatures of 25 and 37°C., the loss in weight being determined after 24 hr. Their surface areas were then approximately measured, and from these values the rate of evaporation per unit area of cuticle obtained. Estimations of surface area were made by cutting the ticks into sections, freeing the pieces of flexible cuticle from blood, and pressing them firmly on to squared paper. The total area was then determined from tracings of the outlines. A female weighing 300 mg. has a surface area of about 1·8 sq.cm.

The results given by eight ticks (two at each saturation deficiency) are shown in Table 4. At 25°C. the rate of evaporation appears to be determined by saturation deficiency, for the water loss per unit of surface at 50 % R.H. is about half that at 0 % R.H. But at 37°C. this relationship no longer appears to hold good, evaporation being some four times as rapid in dry as in half-saturated air. A still more considerable departure is seen if the rates of evaporation at 25 and 37°C. are compared. In dry air the saturation deficiency at 37°C. is no more than double that at 25°C., yet water loss is from five to eight times more rapid. A similar, though less pronounced, effect can be detected at 50 % R.H. Clearly the rate of evaporation increases considerably at the higher temperature. Now it has been shown by Ramsay (1935) that evaporation from the cockroach increases abruptly if the insect is heated above a certain critical temperature (30°C.), and that this effect is associated with the presence of an oily film at the surface of the cuticle which suffers a change of state at the critical temperature and becomes more permeable to water. The increased rate of evaporation from the cuticle of the tick at 37°C. is presumably associated with a similar change.

Table 4. *Evaporation from the cuticle of the engorged female at 25 and 37°C. Two ticks, with the spiracles covered, were exposed to each saturation deficiency*

Rate of evaporation, mg. per cm.² per 24 hr.			
25°C.		37°C.	
0 % R.H. S.D.	50 % R.H. S.D.	0 % R.H. S.D.	50 % R.H. S.D.
23·8 mm.	11·9 mm.	47·1 mm.	23·5 mm.
11·6	6·2	82·1	20·6
10·0	6·7	52·1	22·6

There can be little doubt that evaporation from the cuticle of a tick engorging on a mammalian host, such as a sheep, will normally be considerably influenced by the high temperature of the host's skin. At the base of sheep's fleece, near the point of attachment, the air temperature is usually about 37°C. and the humidity in the region of 50 % R.H. (Davies & Hobson, 1935). Under natural conditions, however, far larger numbers of ticks are found attached among the short hairs of the cheeks, throat and ears. In these more exposed regions the

temperature and humidity gradient from the skin outwards will doubtless be steeper, and the environment, in general, less drying.

Although evaporation from the cuticle may normally be of importance in the elimination of water, other experiments have shown that the normal water content can be maintained even if the humidity is abnormally high. This was shown by engorging a number of ticks in a capsule containing a plaster of Paris and cotton-wool pad which was kept moistened. The air inside the container must have remained saturated with water vapour or nearly so, yet the ticks engorged normally and were found after detachment to have retained only a normal amount of water (about 54 % of the original weight). Under these conditions the Malpighian tubes may perhaps excrete water which would otherwise have been evaporated.

were not covered; loss in weight was therefore due to evaporation from the cuticle and spiracles. The age of the ticks was known, the ixodid females being used about 1 month after the nymphal ecdysis and the *Ornithodorus* nymphs about 1 week after the previous moult.

Fig. 8 shows the daily water loss and length of survival of some typical individuals. The ability of these species to prevent water loss differs strikingly. *Ornithodorus*, *Dermacentor* and *Rhipicephalus* are the most resistant, losing only about 1–3 % of their original weight per diem and surviving 35, 27 and 17 days respectively. *Amblyomma cajennense* loses water at the rate of about 5 % per diem and survives some 10 days. *Ixodes canisuga* and *I. hexagonus* are still less resistant and survive only 4–5 days. *I. ricinus* occupies the lower end of the series, survives only 1 or 2 days, and loses up to half the body weight per diem. Among the slight, although probably significant, specific differences, one notes that *Dermacentor andersoni* is more resistant than *D. reticulatus*, and *Ixodes canisuga* more so than *I. hexagonus*. On the other hand, the behaviour of *Amblyomma cajennense* and *A. maculatum* (not shown) is very similar.

Fig. 8 also shows that, as has already been noted in the case of *Ixodes ricinus*, the rate of water loss nearly always increases when desiccation becomes excessive.

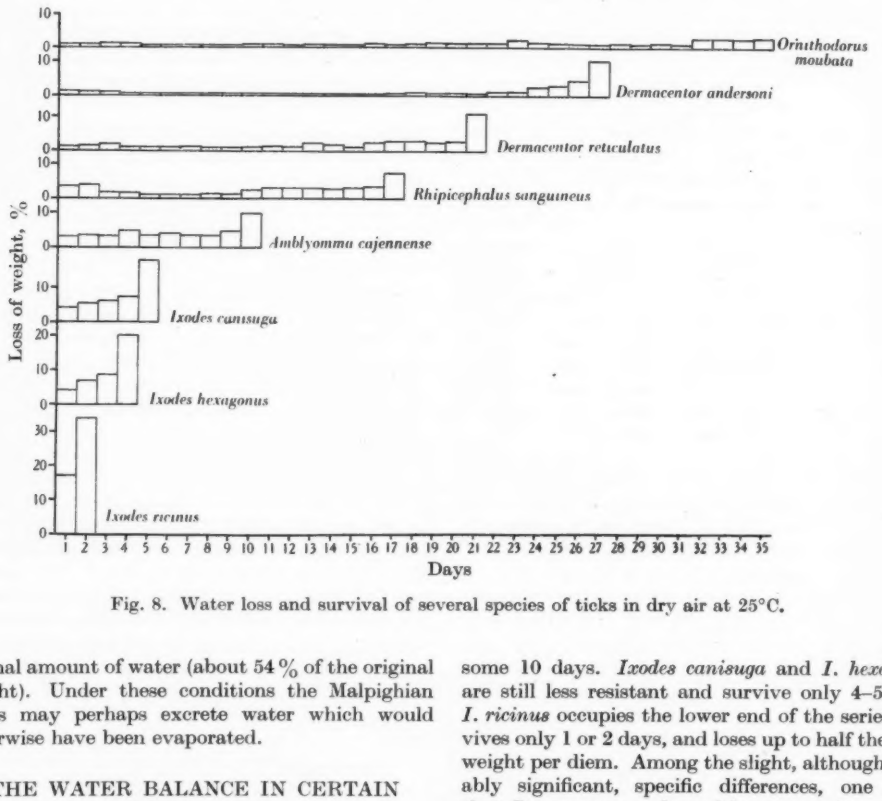

Fig. 8. Water loss and survival of several species of ticks in dry air at 25°C.

THE WATER BALANCE IN CERTAIN OTHER TICKS

(i) *The resistance to desiccation*

The resistance to desiccation of *Ixodes ricinus* and eight other species has been compared; the latter included one argasid tick, *Ornithodorus moubata*. Unfed females (or, in the case of *Ornithodorus*, nymphs of a comparable weight) were exposed to dry air at 25°C. and weighed daily. The spiracles

(ii) *The equilibrium humidity*

Unfed females or nymphs of five of these more resistant species were desiccated for 5 days in dry air, exposed to 95% R.H. for 2 days and then reweighed. The results, given in Table 5, show that all these species gain water at this humidity. The rate of uptake by the ixodid ticks is particularly high and may in fact be more rapid than the rate of loss in dry air. Further evidence on this point is given below.

Table 5. *Weight, in mg., of unfed females of certain ixodid species, and of the nymph of* Ornithodorus, *after desiccation in dry air for 5 days and after subsequent exposure to* 95% R.H. *for 2 days*

	Original weight	Weight after desiccation	Weight after water uptake
Ornithodorus moubata	5·70	5·10	5·32
Dermacentor andersoni	6·30	5·68	6·22
Rhipicephalus sanguineus	2·88	2·52	3·00
Amblyomma maculatum	6·58	5·12	5·90
A. cajennense	7·25	5·70	6·40

Since these other species also lose water at low, and take up water at high humidities, conditions of equilibrium will be realized, as in *I. ricinus*, at certain intermediate humidities. These equilibrium humidities have been determined, to the nearest 2% R.H., by desiccating the ticks in dry air and exposing them to successively higher humidities until water loss ceased and uptake began. The following values were obtained:

Ornithodorus moubata (four nymphs): 82, 84, 86, 90% R.H.

Dermacentor andersoni (three unfed females): 86, 88, 90% R.H.

D. reticulatus (three unfed females): 86, 86, 88% R.H.

Rhipicephalus sanguineus (three unfed females): 84, 88, 90% R.H.

Amblyomma cajennense (three unfed females): 90, 90, 92% R.H.

A. maculatum (three unfed females): 88, 88, 90% R.H.

Ixodes canisuga (three unfed females): 92, 94, 96% R.H.

I. hexagonus (three unfed females): 94, 94, 94% R.H.

It is clear that the equilibrium humidities, which range from 82 to 96% R.H., are comparatively constant in all the species studied. And there is certainly no general correlation between equilibrium humidity and resistance to desiccation. On the other hand, it is true that in *I. canisuga* and *I. hexagonus* the values obtained are slightly and consistently higher than in the other species, a fact which implies that at a given humidity the former are rather less efficient at taking up water.

(iii) *The site of uptake*

Similar experiments to those already described (p. 7) were carried out in order to determine whether *Ornithodorus*, *Amblyomma maculatum* and *Ixodes hexagonus*, species far more resistant to desiccation than *I. ricinus*, also take up water through the cuticle. After an initial desiccation in dry air, the spiracles were covered and the ticks were transferred to a humidity above the previously determined equilibrium humidity. The results were as follows:

Ornithodorus moubata. Five nymphs, after desiccation for 5 days at 0% R.H., were exposed to 95% R.H., the spiracles having been covered. None showed any increase in weight. The blocks were then removed and the ticks returned to 95% R.H. for a further 5 days. There was some loss in weight on the first day, but at the end of this period all showed net increases in weight.

Of five other nymphs treated similarly save that the spiracles were not blocked, four began to increase in weight within 2 days of transfer to 95% R.H. and continued to take up water at an average rate of 1·2% of their original body weight per diem. One failed to take up any water.

Amblyomma maculatum. Five unfed females were exposed to dry air for 3 days, the spiracles were covered and they were transferred to 95% R.H. All gained water immediately after transfer and continued to do so at an average rate of 2·1% of the body weight per diem. Five desiccated ticks with the spiracles free took up water at a mean rate of 2·9% per diem.

Ixodes hexagonus. Five unfed females, desiccated in dry air for 2 days, were removed to saturated air after covering the spiracles. All immediately increased in weight, the mean rate of uptake being 7·3% per diem.

These results show that, provided they do not become asphyxiated, *Amblyomma maculatum* and *Ixodes hexagonus* gain water whether the spiracles are covered or are free, and do so at about the same rate. The site of absorption, as in *I. ricinus*, must then be at the surface of the cuticle. A similar demonstration was not possible in *Ornithodorus*, for no individuals with covered spiracles were ever found to gain water, although they might do so when the blocks were subsequently removed. Although it is conceivable that water is taken up by some part of the respiratory system in this species, a more probable explanation is that when the spiracles are covered oxygen lack interferes with normal secretory activity.

(iv) *A quantitative estimate of the rate of passage of water through the cuticle in different species*

Since all the species of ixodid ticks examined, with some individual exceptions, not only survive, but also retain their normal functional activity when their spiracles are covered, a quantitative comparison of the rates of evaporation and of uptake over a unit surface area of cuticle can be made; and the rates of transfer of water in the direction endo- to epicuticle and in the reverse direction can then be compared in the different species in terms of absolute units.

To carry out the determination, unfed ticks with the spiracles covered were desiccated in dry air for 2 or 3 days at 25°C. and were then exposed either to 95% R.H. or to saturated air. From the changes in weight are obtained the mean rate of evaporation and the rate of uptake per 24 hr. Since the rate of uptake falls off progressively as the normal water content is approached, the water gained during the first 24 hr. after transference to the high humidity was taken as representing this value. The surface area was then approximately estimated. This was accomplished by compressing the tick gently between two glass slides and making a camera lucida drawing of the body outline on squared paper, the area being afterwards read off. Since in shape the body of the unfed tick is quite flat, the total surface area was taken as twice this value. In this very formal estimate, which is merely useful in distinguishing species differing considerably in size such as *Dermacentor* and *Rhipicephalus*, the surface of the sides of the body and of the legs are omitted; but even if these were taken into account, no proper allowance could be made for the corrugated surface of the epicuticle, etc.

The results are shown in Table 6. The rates of evaporation may be compared with the rate of water loss from a free water surface. At 0% R.H. and 25°C. such a surface was found to evaporate water at the rate of 230 mg. per cm.² per 24 hr.

As was to be expected from the previous results, the rate of evaporation from a unit area of cuticle varies considerably in the different species. *Dermacentor andersoni* and *Ixodes ricinus* occupy the extreme positions in the series, the former losing about 0·4 mg. of water per cm.² per 24 hr. at 25°C., the latter as much as 5·6 mg. Even in *I. ricinus*, however, the rate of evaporation is obviously very small compared with that from a free-water surface.

When the rates of uptake, either at 95% R.H. or in saturated air, are compared, these values are seen to be relatively constant in the different species; in other words, the fact that the rate of evaporation is much lower in some species does not imply that water absorption is hindered or even that the rate of uptake is diminished. *Dermacentor*, for example, takes up water at approximately the same rate as *Ixodes ricinus*. The exceptionally low rate of uptake by *I. hexagonus* at 95% R.H. can be attributed to the circumstance that this humidity is only slightly above the equilibrium humidity in this species (p. 15). On the other hand, the behaviour of *Ornithodorus* is somewhat different, for although the equilibrium humidity in this species is as low as in most Ixodidae the uptake of water is much less rapid even when the humidity lies well above the point of equilibrium (Table 5).

Table 6. *The rate of passage of water through the cuticle at* 25°C. *in unfed females of several species of Ixodidae. After desiccation in dry air each individual has been exposed either to* 95% R.H. *or to saturated air. Uptake and evaporation are also expressed as a ratio* (U/E)

	mg. per cm.² per 24 hr.			mg. per cm.² per 24 hr.		
	Rate of evaporation in dry air	Rate of uptake at 95% R.H.	U/E	Rate of evaporation in dry air	Rate of uptake in saturated air	U/E
Dermacentor andersoni	0·5	1·6	3·2	0·3	3·0	10·0
Rhipicephalus sanguineus	1·2	2·7	2·2	1·6	5·1	3·2
Amblyomma cajennense	1·2	1·9	1·6	1·3	3·1	2·4
Ixodes hexagonus	2·4	0·5	0·2	3·8	2·0	0·5
I. ricinus	5·6	1·9	0·3	4·4	4·0	0·9

Evaporation and uptake can be expressed conveniently as the ratio:

$$\frac{\text{Rate of uptake}}{\text{Rate of evaporation}} \text{ or } \frac{U}{E}$$

(Table 6). For those species in which the rate of evaporation is high, this ratio is less than unity, even when uptake is from saturated air. At the other end of the series, in species such as *Dermacentor* and *Rhipicephalus*, the ratio may have a value ranging from 3 to 10: that is to say, the rate of uptake through the cuticle in saturated air may be from three to ten times as rapid as the rate of evaporation in dry air.

DISCUSSION

The maintenance and regulation of the water balance

The exchange of water in *Ixodes ricinus* takes place almost exclusively through the cuticle. The respiratory system plays relatively little part in the exchanges, indeed, covering the spiracles has no appreciable effect either on uptake of water at high or on evaporation at low humidities. Since, in addition, the adjustment of this balance in the unattached stages does not involve the excretion of water by the Malpighian tubes, regulation is evidently achieved solely by the activity of the epidermal cells: this and the ability to retain water must then be attributed to the physical properties of the epicuticle and to the functional activity of the epidermis (cell bodies and pore canals).

The retention of water at low humidities is probably due in part to a film of lipoid overlying the epicuticle. The characteristic effect of temperature on evaporation from the cuticle suggests that such a layer is present. Indeed, the formation of an oily exudate at the surface of the cuticle in the engorged tick can be observed readily, but what effect this secretion has on the permeability of the cuticle has yet to be decided. The similar rates of water loss from a unit area of cuticle in the unfed and engorged tick, and the low rate of loss as compared with a free-water surface, implies that a waterproofing layer must be present in the unfed stages.

The striking differences in the rates of evaporation from the cuticle of the species examined here, and therefore in the ability of these species to survive desiccation, may reflect specific differences in the waterproofing qualities of this lipoid component.

The dark body colour mutants of *Drosophila* have been found to be more resistant to desiccation than those with a normal or yellow colour, and it has been suggested that impermeability is a significant consequence of sclerotization (Kalmus, 1941). From the study of the present series of species, however, it is clear that resistance to desiccation has no connexion with the degree of sclerotization. Thus, for example, the male ixodid with the endocuticle nearly uniformly sclerotized is no more resistant than the female with a soft and colourless endocuticle. Similarly, although the epicuticle in *Ixodes ricinus* is tanned and that of *I. hexagonus* and *I. canisuga* untanned, *I. ricinus* is considerably less resistant than *hexagonus* or *canisuga*.

The manner in which the desiccated tick gains water from humid air is still obscure. The possibility that water is first taken up hygroscopically by the cuticle, and is subsequently withdrawn by the epidermal cells, is worth some initial consideration. According to such a view water could be withdrawn from the larger capillaries of this hygroscopic component, but not from small capillaries where secretion would fail to overcome capillarity. In capillaries of an intermediate size the forces of secretion and capillarity would be balanced, and the vapour-pressure reduction in such capillaries could then be pictured as representing that humidity at which the tick attains a state of equilibrium. Such an hypothesis would account for the dependence of uptake and retention of water near the point of equilibrium on the relative humidity. In spite of some attractions, however, this hypothesis is shown by calculation to be untenable in its simplest form, for capillaries of a diameter giving a vapour-pressure depression corresponding to that of the equilibrium humidity are of such a size that the capillary forces could not be balanced by osmotic forces of the order normally associated with active processes.* But whatever explanation is offered, the uptake of water from high humidities must involve secretory activity. For if, by analogy with other arthropods, the tick haemolymph is assumed to have an osmotic pressure equivalent to a 1% solution of sodium chloride, this will have a relative humidity of about 99·4%. Yet, as we have seen, the tick may be able to gain water from a relative humidity of 90% or even from lower humidities. That uptake from humid air involves secretory activity is also shown by its failure when the tick is temporarily asphyxiated.

In view of the foregoing argument, any attempt to define the mechanism of uptake in more concrete terms would appear to be unprofitable at present: rather can we regard it as an example of secretory activity where the tissue or organ is engaged in taking up and concentrating some particular substance, in this case water, against a concentration gradient. A parallel instance is the active uptake of salts from very dilute media by many fresh-water organisms (Krogh, 1939). The regulation of the water balance in *Ixodes* may now be considered in terms of this concept, that is, as an active process controlled by the epidermal cells and taking place through the cuticle.

The ability of the unfed tick to oppose or to prevent evaporation cannot be attributed entirely

* Consider the example of a tick in equilibrium with 90% R.H. From the well-known expression relating the vapour-pressure depression at the air-water surface in a capillary and the curvature of the meniscus, it is found that a relative depression of 10% is given by a capillary approximately 210A. in diameter. The suction pressure required to draw water from such a capillary is of the order of 140 atm. The osmotic pressure of the tick haemolymph, although unknown, is not likely greatly to exceed that of a 1% solution of sodium chloride. Now such a solution has an osmotic pressure of about 8 atm. In order to withdraw water therefore, the epidermal cells would have to overcome a suction pressure of about 132 atm.

to the physical properties of the epicuticle, for when the tick is injured through excessive desiccation the rate of water loss from the cuticle increases. And this is also clearly shown by the failure to retain water after asphyxiation (p. 7) and after poisoning with hydrogen cyanide. Evidently the loss of water at low humidities is opposed by the secretory activity of the epidermal cells. If now the humidity is progressively raised, a point is reached—the equilibrium humidity—at which evaporation is completely prevented; and if the humidity is again raised, water is taken up. The equilibrium humidity is clearly a point of unstable equilibrium and it requires only small changes, in secretory activity or in external humidity, to shift the balance in either direction.

At humidities above the point of equilibrium water is only taken up so long as the water balance is depleted, secretion ceasing when the normal content has been restored. A more stable state is achieved when the tick reaches equilibrium in saturated air. The absence of secretory activity and the state of adaptation of the epidermal cells is then seen when the tick is transferred from saturated air to a humidity which, although lower, is still above the equilibrium humidity (e.g. 95% R.H., see p. 2), for water is then evaporated until secretory activity develops and opposes further loss. The same effect is of course seen when the tick is transferred from saturated air to a humidity below the point of equilibrium, but in this case evaporation cannot be completely prevented although the rate of evaporation may be reduced. This phenomenon is responsible for one type of irregularity affecting the rate of water loss.

The uptake of water by the desiccated tick is normally delicately adjusted and only the required amount of water is taken up. Sometimes, however, after extreme desiccation, uptake from saturated air is so rapid that excess water is gained. Such imperfect regulation, or over-compensation, is characteristic of secretory processes in general and is best shown when the organism is transferred abruptly from a low to a high concentration. If the tick is gradually exposed to lower humidities, the retention of water does not appear to become more efficient; in other words, secretory activity rapidly reaches its maximum efficiency. Slight changes in efficiency nevertheless remain as a further possible cause of fluctuations in the rate of evaporation.

In accounting for the effects of contact with water there are presumably two main factors to be taken into consideration, namely, the osmotic entry of water and the activities of the epidermal cells. As is to be expected, since osmotic and secretory forces are acting in conjunction, the unfed tick takes up water after desiccation even more rapidly than it does in saturated air and often fails completely to control its entry. This failure is no doubt due in the first place to the rapidity of uptake for, as we have seen, some over-compensation may take place even in saturated air before the epidermal cells cease to secrete water. In those cases where uptake continues unchecked it is conceivable that the cells receive an injury impairing their ability to keep water out. That some such injury, perhaps through the washing out of salts, etc., from the cuticle, follows prolonged exposure to water, appears likely from the inability of ticks so treated to retain water in saturated air. Often, however, the rate of gain of water by the desiccated tick declines after the first day or so, although the entry of water is never entirely stemmed. Uptake by the normal tick in contact with water is also slow. Since the epidermal cells actively eliminate water when the tick is exposed to saturated air after the absorption of excess water, it is possible that osmotic uptake is here opposed by active forces and that the low rate of gain is not merely due to the absence of secretory activity.

The abilities of the engorged stages to regulate the water content are more limited. Thus, engorged nymphs after desiccation are as a rule unable to gain water from humid air, and are consequently in equilibrium only in saturated air. The underlying reason for this difference is uncertain, but it could be pointed out that when the tick engorges the epidermal cells and pore canals change their function and pass from a 'resting' condition to one of great activity in the synthesis of new cuticular material.

The mode of regulation of the water balance in *Ixodes* bears a considerable resemblance to the regulation of the haemolymph chloride in the mosquito larva (Wigglesworth, 1938). The ability of the mosquito larva to take up and to retain chloride in dilute media and to keep it out in concentrated media finds a direct counterpart in the present results. Indeed, the analogy is even closer, for the adaptations developed by the mosquito larva, revealed by the fall in haemolymph chloride when the (adapted) larva is transferred from a high to a low concentration, and over-compensatory changes, such as the exaggerated rise in chloride when the reverse change is made, are very similar to the adaptations displayed by the tick and to the changes in the water content after exposure to the appropriate low or high humidities.

The possible effect of the lipoid layer of the epicuticle on the uptake of water from high humidities has not yet been considered. If the presence of this layer diminishes the rate of evaporation from the cuticle it might be expected that the rate of entry of water would be correspondingly reduced. But no such effect has been found. On the contrary, although the different species examined differ considerably in their powers of limiting water loss, the rate of uptake per unit surface of cuticle at humidities above the point of equilibrium is relatively

constant. This suggests that the factor limiting uptake is the ability of the epidermal cells to secrete water rather than the nature of the epicuticular lipoid. On the other hand, it is possible that the structural and physical properties of the cuticle as a whole are of importance in determining the relative rates of transfer of water across the cuticle. It has been known for many years that certain 'double' membranes of natural and artificial origin may display anomalous behaviour with regard to the rate of transfer of water across them. Hurst (1941) has recently drawn attention to the possible relationship of the asymmetrical structure of insect cuticle to differences in the rates of flow across it. He finds that in isolated blowfly cuticle the rate of diffusion from lipophilic to hydrophilic component (that is, from epicuticle to endocuticle) is far more rapid than in the reverse direction. The remarkable asymmetry shown by such forms as *Dermacentor* and *Rhipicephalus*, which gain water in saturated air up to ten times as rapidly as they lose it in dry air, may imply that similar considerations hold good in these living arthropods. On the other hand, the precise effects of asymmetry, if any, cannot be assessed properly in the present case for they are concealed by the operation of other active processes also profoundly influencing the transfer of water across the cuticle.

This study of the water balance in *Ixodes ricinus* has shown that the tick can best be regarded as an organism in a state of dynamic equilibrium with the external medium, in this case the humidity of the air. The physical nature of the cuticle serves only to retard the rate of loss of water and so brings it more closely within the range of the active processes which effect the delicate adjustments. Evaporation from the surface of a living arthropod may evidently be a complex phenomenon—one which cannot be adequately defined in terms of evaporation from a simple membrane, more or less permeable as the case may be, and of the external humidity, however this is expressed. For as well as these factors we may also have to take into consideration the physiological state and functional abilities of the organism.

The water balance in relation to the normal environment

Unless permanently sheltered by a humid microclimate, the survival of the terrestrial arthropod will depend to a considerable extent on its powers of limiting evaporation. This is no doubt true of the majority of species we have been concerned with here, particularly as the fasting tick must often spend a lengthy period awaiting a suitable host. Nevertheless, in order to preserve the water balance —and in the absence of other sources of water—the unfed tick does not merely rely on resisting water loss, for if the humidity rises above the point of equilibrium any water loss can be made good. Water gained from humid air probably forms the most important, and perhaps the sole source of water for the unfed tick in nature. Uptake will occur if the tick enters a humid microclimate; or it may take place during extreme diurnal fluctuations in the humidity, such as normally occur as the air cools. The engorged stages, we have seen, are as a rule unable to take up water from humid air. One suspects, however, that by far the larger part of the life span of most species of Ixodidae is usually spent in the unfed state.

The distribution of *I. ricinus* is known to be confined to areas of rough grazing where the vegetational conditions are such as to provide a 'mat' of moist, decaying plant material or where the ground is permanently wet. It is clear that in the 'mat' or at the base of the growing herbage the air humidity will remain high and may indeed normally approach saturation. Macleod (1936) and Milne (1944) have concluded that the tick is able to survive in these localities only by virtue of this microclimate. The present findings are entirely in harmony with this suggestion, for, of the eight species of ixodid ticks, several of them inhabiting environments where the humidity conditions would be expected to be more rigorous, *I. ricinus* is the least resistant to desiccation.

The engorged stages of *I. ricinus* are found at the base of the growing vegetation or perhaps actually in the 'mat' where this is present. If, then, the conditions are generally favourable for survival, it is unlikely that the engorged tick will ever be exposed to a large saturation deficit. The unfed stages probably also take up a similar position during the greater part of the year, but, on becoming active, climb upwards preparatory to attaching to a host. They may leave the zone of saturated air and will 'experience temperature and humidity conditions approximating to those of the macroclimate' (Milne, 1944). These conditions have recently been analysed in detail with the aid of thermohygrograph records taken over a period of 2–3 years on two heavily infested farms (Milne, 1945). It is noteworthy that during periods of tick activity the air humidity rarely falls below 50% R.H., even at midday, and at night commonly approaches saturation, rising, that is, to a value well above the equilibrium humidity of the unfed tick. Nevertheless, from the figures given by Milne it seems unlikely that the tick would be able to maintain its water balance while remaining at the vegetation tips; rather does it appear more probable that it restores the balance by returning to the base of the vegetation and so re-entering the zone of saturated air.

SUMMARY

The unfed tick gains water from humid air or from water in contact with the cuticle, and loses water by evaporation. Whilst attached to the host the tick is gaining water from the ingested blood and losing

water in the excrement. The engorged tick usually lacks the ability to take up water from humid air.

The exchange of water takes place mainly through the cuticle. Regulation of the water balance is therefore brought about by the activity of the epidermal cells.

The cuticle comprises two principal layers, the epicuticle and endocuticle. The epicuticle is overlaid by a lipoid possessing important waterproofing properties. The pore canals, which traverse the endocuticle, are occupied by cytoplasm, and may in consequence play an important role in the active transfer of water through the cuticle: they do not penetrate the epicuticle.

Water loss from the unfed tick is not closely related to saturation deficiency, particularly at high humidities. This departure is due to a physiological cause, namely, to the ability to secrete water. The effects of this activity are such that a state of equilibrium is attained at a relative humidity of about 92%: at lower relative humidities the tick loses water by evaporation, while at higher humidities it takes up water. The retention of water at humidities below the point of equilibrium is due not only to the physical properties of the epicuticle but also to this secretory activity, for water loss increases when the tick is temporarily asphyxiated, poisoned with cyanide or injured through excessive desiccation. Near the point of equilibrium the loss or gain of water over a wide range of temperature is determined by the relative humidity.

The uptake of water from humid air occurs when the tick is in a desiccated condition but ceases as the normal water content is restored. After previous exposure to saturated air the adapted tick at first loses water at relative humidities above the point of equilibrium, but later comes to retain water completely.

Both unfed and engorged ticks possess the ability to prevent or to limit temporarily the entry of water in contact with the cuticle.

The engorging female, originally weighing about 2 mg., ingests about 600 mg. of blood. About 300 mg. or two-thirds of the contained water are usually eliminated before the end of engorgement. Evaporation from the cuticle may account for a considerable fraction of this, for the temperature to which the attached tick is exposed (about 37° C.) is, in *Ixodes ricinus*, above that temperature at which a marked increase in the permeability of the epicuticular lipoid takes place.

The nine species of ticks examined differ considerably in their powers of limiting evaporation. This may reflect specific differences in the nature of the epicuticular lipoid. The order of their resistance is as follows: *Ornithodorus moubata*; *Dermacentor andersoni*; *D. reticulatus*; *Rhipicephalus sanguineus*; *Amblyomma cajennense* and *A. maculatum*; *Ixodes canisuga*; *I. hexagonus*; *I. ricinus*. In dry air water loss through the cuticle is 10–15 times more rapid in *Ixodes ricinus* than in *Dermacentor andersoni*. The more resistant species also take up water through the cuticle after desiccation; indeed, the rate of uptake over a unit area of cuticle is approximately the same in all species of Ixodidae. Uptake thus appears to be limited by the ability of the epidermal cells to secrete water.

Stocks of *Dermacentor andersoni*, *Rhipicephalus* and *Amblyomma* spp. were kindly supplied by Dr R. A. Cooley, Director of Entomology, Rocky Mountain Laboratory, U.S.A., through the good offices of Prof. P. A. Buxton, F.R.S. I am also indebted to Dr H. H. Green of the Veterinary Laboratory, Weybridge, for providing useful facilities, and to Dr V. B. Wigglesworth, F.R.S., for his generous help throughout the various stages of this work.

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TOBACCO ROSETTE: A COMPLEX VIRUS DISEASE

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(With Plates I and II)

INTRODUCTORY

In 1938 the writer received through the courtesy of Dr J. C. F. Hopkins, Senior Plant Pathologist, Southern Rhodesia, a piece of tobacco stem with some leaves attached. It was suggested by Dr Hopkins that the tobacco was affected with a virus disease. Aphides of the species *Myzus persicae* Sulz. were colonized on the tobacco, with some difficulty since the specimen was very faded, and then transferred to healthy tobacco seedlings, some of which developed a virus disease for which the name *tobacco rosetté* has been suggested. At the same time Wickens (1938), working independently in Southern Rhodesia, also transmitted the disease by means of *M. persicae* and he has published a preliminary account of it. In 1939 the writer received another specimen of tobacco from Mr Colin Smee in Nyasaland, and investigation showed that it also was infected with rosette.

In the present paper an account is given of the complex nature of the disease, the symptomatology of the component viruses, alone and in combination, the histopathology of the rosette disease and a preliminary statement on the methods of transmission of the viruses. A detailed study of the insect relationships will be published separately.

COMPLEX NATURE OF THE ROSETTE DISEASE

That rosette was caused by more than one virus was discovered early in the work when attempts were made to transmit the disease by sap inoculation. The symptoms resulting in tobacco plants, var. White Burley, when inoculated mechanically from a rosetted plant, differed greatly from the symptoms produced by aphis transmission from the same source (Pl. I, figs. 1, 3). It was thus demonstrated that tobacco rosette was caused by two independent viruses, only one of which was sap-transmissible. Both viruses, however, were carried very efficiently in combination by the aphis *M. persicae*.

The virus, which is both sap- and aphis-transmitted, has been provisionally called the *mottle* virus to distinguish it from the other component of the rosette complex which is aphis-borne only and for which the name *vein-distorting* virus has been suggested. As its name implies the chief symptom of the first-named virus is a mottling, while the second virus induces curvature of the veins of the leaves with some stunting of the plant. Neither of the viruses singly produces the gross distortion of the plant characteristic of the double infection (Pl. I, fig. 1).

It is of interest to note that the specimen of tobacco received from Nyasaland also contained the same two viruses. It is not known whether the rosette disease investigated by Wickens was the same complex, but his photographs of the disease strongly suggest that this was so.

SEPARATION OF THE COMPONENT VIRUSES

Isolation of the mottle virus from the rosette complex was, of course, easy enough. Sap, extracted from a rosetted plant, and inoculated by gentle rubbing to White Burley tobacco seedlings, gave a high percentage, usually 100 %, of infection with the mottle virus alone.

The isolation of the vein-distorting virus was not so easy, since all attempts to separate it from the complex by the use of different species of aphides and by differential feeding methods were at first unsuccessful. Transmission experiments were then carried out to different Solanaceous hosts in the hope of finding one which would be susceptible to the vein-distorting virus but immune to the mottle virus. These attempts to find a filter plant have so far proved unsuccessful, since all the species tried were equally susceptible to both viruses. Finally, the separation was achieved by chance, in a series of transmissions by the aphis a case occurred in which the insect transmitted the vein-distorting virus but omitted the mottle virus.

THE COMPONENT VIRUSES

The mottle virus

The first sign of infection in tobacco, var. White Burley, is a rather pronounced 'clearing' of the veins in the youngest and next youngest leaves, a common primary symptom in mosaic-type viruses, and it develops from 7 to 10 days after infection at a mean daily temperature of about 70° F. At the

same time there is a tendency for the smallest and youngest leaf to bend downwards and inwards (Pl. I, fig. 2), and this is a point of similarity to the development of the rosette disease except that in rosette this downward curling is more or less permanent while in the mottle disease the leaf straightens out again as growth continues.

As the plant develops, the vein clearing disappears and a rather bold dark green mottle takes its place, consisting of large irregular blotches of dark green upon a lighter green background. Necrotic spots are frequently present. No vein banding such as occurs with some mosaic viruses, notably potato virus *Y*, has been observed. As the tobacco plant gets older the symptoms tend to get fainter and may almost disappear. On Turkish tobacco the symptoms are very similar.

Datura Stramonium reacts in a similar manner to tobacco though the symptoms may be rather more severe. Vein clearing develops, followed by mottling and some distortion and twisting of the young leaves. As the plant grows these symptoms fade somewhat leaving only a faint mottling of the leaves. On tomato the symptoms are not very pronounced, consisting of a faint preliminary vein clearing followed by a transient mottling of the leaves. So far the virus has not been transmitted to any plant outside the Solanaceae.

Description of the mottle virus

The properties of this virus seem very similar to those of many insect-borne, sap-transmissible viruses. The thermal inactivation point is low, about 55° C., the longevity in extracted sap is only about 2 days and the dilution end-point is about 1 : 100.

The vein-distorting virus

The symptoms caused by this virus differ from those of the foregoing in that there is no clearing of the veins and no mottling. The manifestations are purely distorting and in tobacco show mainly in the leaf veins which are not only decidedly crooked but may also curl downwards, particularly at the leaf apex, making the leaf tips arch in a characteristic manner. The first sign of the disease, which occurs about 10 days after infection, takes the form of a bending or crumpling of the central leaf. As this leaf grows the edges curl inwards on both sides so that the leaf assumes almost a tubular appearance (Pl. I, fig. 4). This is followed by a slight bulging of the interveinal tissues and the commencement of the characteristic distortion of the veins. On other Solanaceous plants the symptoms are essentially the same. It has not been found possible to transmit this virus to plants outside the Solanaceae. Since the vein-distorting virus is not sap-inoculable, there is no information at present on its properties.

Symptomatology and histopathology of the virus complex

The most extreme manifestations of the rosette disease, caused by the two viruses together, occur in the tobacco plant, var. White Burley, and these are described in some detail. The first sign of infection is similar to what occurs with mottle virus alone, i.e. clearing of the veins, and this is followed almost immediately by the curling inwards of the youngest leaves. In this case, however, the inward curling persists, and as new leaves are formed they also curl inwards, the resulting rosette getting tighter and tighter until the centre of the plant consists of a ball of compressed leaves with the interveinal tissue bulging outwards. At this stage the rosette assumes a characteristic pallor (Pl. I, fig. 1). When a young tobacco seedling is infected the plant remains as a tight rosette for several weeks, but an older plant, already growing upwards, responds to infection with a rosette at the end of the stem and growth ceases for the time being.

After a tobacco plant has been in the rosette condition for some weeks, upward growth commences, and as it proceeds the tissues commence to split. These splits or fissures develop longitudinally in the stem (Pl. I, fig. 6), in the petioles, in all the veins of the leaves and even in the flowers, most of which fall without opening. A histological examination has been made of these fissures and an account is given in a subsequent paragraph. The splitting of the tissues is most pronounced in the tobacco, but it also occurs to a less degree in *Nicotiana sylvestris* and the tomato plant.

As the tobacco plant continues to grow, a third symptom sometimes, but not invariably, develops. This is the production of outgrowths—enations—on the under-surface of the leaves (Pl. I, fig. 5). The enations vary in size from a few millimetres in diameter to growths which are almost equal in size to the normal leaf. These enations have only been observed in the tobacco plant.

The tomato plant is fairly easily infected with the rosette complex if colonized with infective aphides while in the seedling stage. The symptoms differ from those in the tobacco as there is no rosetting. The leaves of the infected tomato assume a characteristic pallor, somewhat reminiscent of potato leaf-roll, and there is a certain amount of twisting and distortion, but the upward growth of the plant is not apparently affected. Some splitting of the tissues has been observed. The fruit has also been observed to split on occasions, but it is not certain that this was directly due to the virus in-

fection. In *Nicandra physaloides* the main symptoms are pallor and harshness of the leaves, cessation of growth and slight rosetting of the growing point. *Atropa belladonna* reacts with some leaf pallor and distortion, but this later disappears and the viruses are apparently carried without symptoms. Splitting has not been observed in these two species.

Histopathology of the rosette disease in tobacco

In order to investigate the reasons for the splitting of the tissues in tobacco plants affected with the rosette complex, tissues were embedded in wax and sections were cut. The material was fixed in chrome-acetic or formol-alcohol, and the sections were stained in Heidenheim's haematoxylin and safranin. The photomicrographs were taken with a Zeiss camera attachment, using a No. 4 eyepiece and $\frac{2}{3}$ and $\frac{1}{6}$ in. objectives. The best results were obtained by selecting a piece of young stem just before, or just at the commencement of splitting. By means of a hand lens suitable stems could be selected. Examination of a large number of sections suggests that the splitting starts in the cambium and in the unlignified xylem, passes through the phloem, xylem and cortex, and so reaches the exterior. At the same time there seems to be a certain amount of pull exerted on the epidermis causing it to turn inwards. This is probably due to stresses set up in the tissues just beneath. It is suggested that there is a concentration of virus in the cambium which prevents the formation of the normal xylem. Abnormal tissue and giant cells are formed in the cortex and pith. This appears to set up stresses which cause the splitting. Pl. II, fig. 7 is a photomicrograph of a transverse section through a young tobacco stem just at the commencement of splitting. In the centre of the photograph can be seen a slight depression in the epidermis which is the beginning of a split. Just to the right of this appear a number of elongated cells; it is at about this point that stresses seem to be set up which appear to pull in the epidermis. To the right of this can be seen the first development of necrosis, appearing black in the photograph, and to the right of this again is the abnormal tissue beginning to develop inside the vascular system. Pl. II, fig. 8 is a photomicrograph taken at a slightly later stage in the development of the splitting. The fissure has not yet split open, but the epidermis has been dragged down farther towards the focus of necrosis which has enlarged. Pl. II, fig. 9 is a section through a petiole in which a fissure is developing. It shows the inhibition of xylem formation and the abnormal cells. At the top of the photograph can be seen the encroaching fissure. Pl. II, fig. 10 is a section through a normal petiole of the same age as that shown in fig. 9 and similarly treated.

Transmission of the viruses

As already stated only the mottle virus can be transmitted by sap inoculation, and this virus is easily spread by the ordinary methods of mechanical transmission. Both viruses are aphis-borne, and the whole complex is regularly transmitted by the aphis *Myzus persicae* Sulz. At the start of the work on insect transmission it was rather rare for the insect to drop out one of the two component viruses of the complex, but when it did the virus dropped was the vein-distorting virus. In long series of twenty serial transfers in which the infective aphis was moved at 24 hr. intervals to fresh White Burley tobacco seedlings, infection with both viruses persisted to the end, but individual plants occurred in the series which were infected with the mottle virus alone. It was not until much later that the aphis began to drop out the mottle virus and so enabled the vein-distorting virus to be obtained in pure culture.

It is clear that in their relationship with the aphis vector both viruses are of the 'persistent' type (Watson & Roberts, 1939), the insect retaining infection for long periods without again having access to a source of virus. This is shown by the following experiment. Aphides which had fed on a rosette tobacco plant for some days were colonized, on 9 August, on a cabbage plant which is immune to the rosette complex. On the following day and afterwards at daily intervals till the 16th, nine aphides were transferred from the cabbage to three tobacco plants, three aphides per plant. This made twenty-one tobacco plants, all of which developed the complex rosette disease.

M. persicae is not a specific vector of the rosette disease, since successful transmissions have been obtained with *M. pseudosolani*. This aphis, however, appears to be less efficient as a vector than *M. persicae*. *Macrosiphum gei* has also been tried with rather uncertain results.

Seed transmission

There is no evidence that either of the viruses is carried in the seed. Owing to the tendency of the flowers of rosetted tobacco plants to fall without opening, it is difficult to obtain seed from infected plants. However, a certain amount of seed was finally obtained from tobacco, tomato and *Nicotiana glutinosa* plants infected with rosette, and this was planted. All the plants obtained were free of both viruses.

Effect of adding other viruses to the vein-distorting virus. A few experiments were carried out to compare the effect of adding other sap-transmissible viruses to tobacco plants infected with the vein-distorting virus alone. This was done in order to find out whether any of the new complexes would

cause either the peculiar splitting of the tissues previously described or the intense rosetting. The viruses used were potato virus *Y*, an undescribed virus from potatoes known as veinal necrosis virus, *Hyoscyamus Virus* 1, and cucumber mosaic virus. In none of these complexes did splitting develop nor was there any rosetting. The component viruses in each complex seemed to retain their own characteristic symptoms, and there was no merging of the two as is the case with the original disease.

SUMMARY

An account is given of a composite virus disease of tobacco for which the name *tobacco rosette* has been suggested.

The two component viruses, named the *mottle* and *vein-distorting* viruses respectively, have been separated, and their symptomatology and methods of transmission described. The mottle virus is both sap- and aphis-transmitted, but the vein-distorting virus is aphis-borne only.

The symptoms and histopathology of the complex disease in the tobacco plant are dealt with in some detail. There are three main types of symptoms: (1) intense rosetting, (2) splitting of the tissues, (3) formation of enations on the under-surface of the leaves.

The splitting of the tissues has been examined microscopically, and a number of photomicrographs are given illustrating the formation of the fissures. It is suggested that there is a concentration of virus in the cambium which prevents the formation of the normal xylem. Abnormal tissue and giant cells are formed in the cortex and pith. This appears to set up stresses which cause the splitting.

The insect vector of the complex disease is the aphis *Myzus persicae* Sulz. Another aphis, *M. pseudosolani* Theob., is also a vector but is less efficient than *M. persicae*.

The writer's thanks are due to Prof. F. T. Brooks, F.R.S., with whom he discussed the histopathology of the rosette disease, to Dr Roy Markham for taking the photographs illustrating Pl. I, figs. 2–5, and to Mr Charles Harpley of the Molteno Institute for his assistance in taking the photomicrographs.

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EXPLANATION OF PLATES

Plate I

Fig. 1. Young White Burley tobacco infected with the composite disease in the rosette stage. Note the compact rosette with the characteristic pallor.

Fig. 2. Primary symptoms of infection with the mottle virus, young White Burley tobacco ten days after infection. Note the curling of the central leaf and clearing of the veins.

Fig. 3. Secondary symptoms caused by the mottle virus on White Burley tobacco.

Fig. 4. Primary symptoms caused by the vein-distorting virus on White Burley tobacco. Note the turning in of the leaf edges.

Fig. 5. A large outgrowth or enation on the underside of a tobacco leaf affected with the rosette disease.

Fig. 6. White Burley tobacco plant in the later stage of the rosette disease. Note the splits in the stem and the split running longitudinally in the under-surface of the petiole.

Plate II

Fig. 7. Transverse section through a young stem of White Burley tobacco infected with rosette, made just as a split was beginning to develop. Note slight depression in epidermis, necrosis and formation of abnormal tissue.

Fig. 8. Transverse section through a similar stem at a slightly later stage in the development of the split, the fissure is now much deeper.

Fig. 9. Transverse section through a petiole with a developing split, note the inhibition of xylem formation, abnormal cells and fissure at the top of the photograph.

Fig. 10. Transverse section through normal petiole of the same age.

Figs. 7 and 8 were photographed with no. 4 eyepiece and $\frac{2}{3}$ in. objective, figs. 9 and 10 with no. 4 eyepiece and $\frac{1}{6}$ in. objective.

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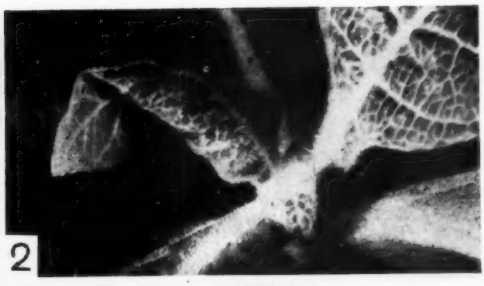

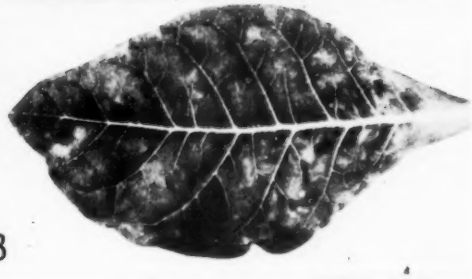

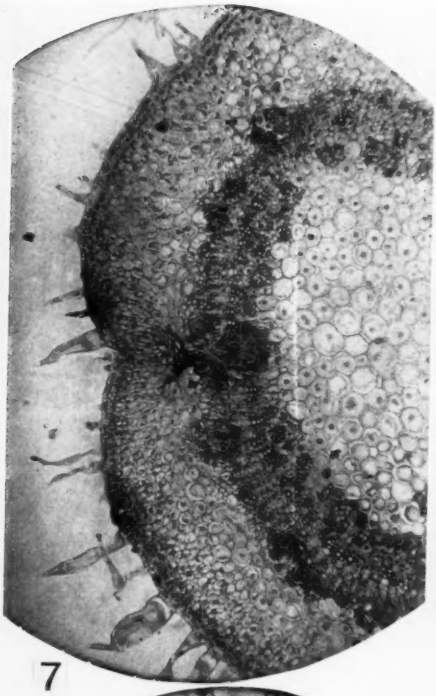

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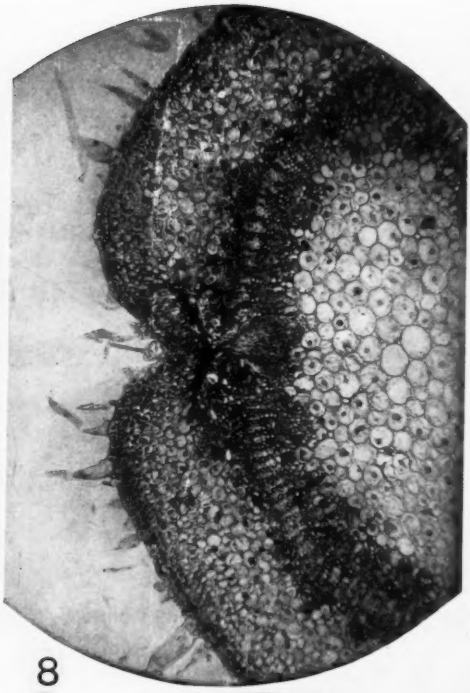

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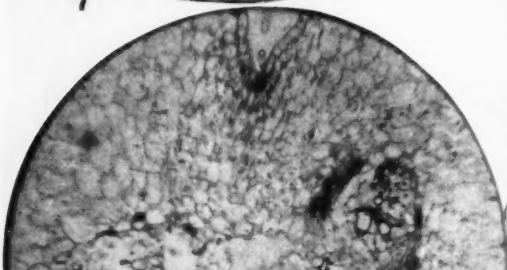

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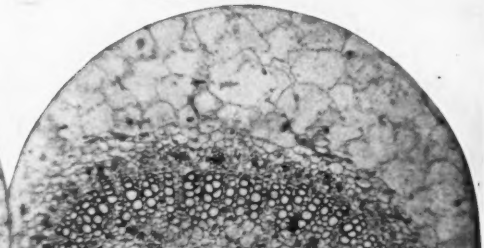

10

THE TRANSMISSION OF PLANT VIRUSES BY APHIDES

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(With 7 Figures in the Text)

In a previous communication an account was given (Smith, 1945) of a complex virus disease of tobacco for which the name *tobacco rosette* was suggested. The components of the complex were named respectively the *mottle* and *vein-distorting* viruses.

In the present paper an account is given of experiments on the transmission of this virus complex by means of the aphis *Myzus persicae* Sulz., and in the second part of the paper a detailed analysis is made of similar experiments carried out by other workers using different viruses.

PART I. EXPERIMENTS ON VEIN-DISTORTING AND MOTTLE VIRUSES

Material and methods

The stock cultures of *Myzus persicae* were kept, for the most part, on non-solanaceous hosts to avoid accidental contamination with the viruses under study, which appear to be confined to the Solanaceae. The plants on which the aphides seemed to thrive best were Chinese cabbage, radish, turnip and spinach.

In the 1943–4 experiments all the aphides used were derived from a single aphis culture, but this was not the case with experiments carried out in previous years.

The plants used in the infections were exclusively small tobacco seedlings, var. White Burley. The insects were placed on the experimental plants and covered with a lamp chimney or glass tumbler and allowed to feed for the necessary period. They were then removed from the plants with a camel's hair brush, and the plants were sprayed with nicotine and kept under observation.

Results

Table 1 shows the result of an experiment in which aphides were fed on a rosette-infected plant for several days, and then transferred daily on to a series of healthy plants, using one aphis per plant.

Table 1. *Twenty daily transfers of six aphides, one aphis per plant (October 1938)*

	Transfer																		
Aphis	1	2	3	4	5	6*	7	8	9	10	11	12*	13	14	15	16	17	18*	19	20
1	*R*	*R*	*M*	*R*	*R*	Aphis died														
2	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*M*	*M*	*R*	*M*	*R*	*R*	*M*	–	*R*	*M*	*M*
3	*R*	*R*	*R*	*R*	*R*	*R*	*M*	*M*	*R*	*M*	*M*	*R*	*M*	*R*	*M*	*M*	*R*	*R*	*R*	*M*
4	*M*	*M*	*R*	*R*	*R*	*R*	*R*	*M*	*M*	*R*	*M*	*M*	*M*	*R*	*R*	*R*	*M*	*R*	*R*	*R*
5	*M*	–	*R*	*M*	*M*	*R*	*R*	*R*	*R*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	–	*R*	*R*	*R*
6	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*R*	*M*	*R*	*R*	Aphis died							

* Feeding period on healthy plant 48 hr. instead of 24 hr.
R = infection with the complex disease, rosette.
M = infection with mottle only.
– = no infection.

Six aphides were used, and twenty serial transfers were made.

Usually the infections obtained consisted of the combination disease rosette (*R*), less frequently of the mottle disease (*M*). It is evident that both viruses are able to persist in the aphis for over 3 weeks.

The results of further experiments are summarized in Table 2. The source of infection was in most cases a plant infected with the complex disease rosette (*R*), in other experiments a plant infected with the vein-distorting virus (*V*) only. Two similar experiments using as the source a plant infected with the mottle virus only failed to give any infections, and are not included in Table 2. Further tests confirmed the interesting discovery that the aphis is apparently unable to pick up the mottle virus when this exists alone in the plant. It seems to be necessary that the vein-distorting virus is also present to allow

the mottle virus to be insect-borne. There is at present no very obvious explanation of this phenomenon, but it is under investigation and the results will be published separately.

In experiments using the rosette source, some of the healthy plants became infected with the rosette complex, and some with mottle virus only. For the purpose of Tables 2–5, transmission of either the rosette complex, or of mottle alone, is regarded as a positive result.

Table 2 shows that after periods of 10 min., 1 hr. or 24 hr. infection feeding, the insects are less infective during their first 48 hr. on healthy plants than subsequently, showing that with these viruses there is a 'latent' period such as has been demonstrated with other persistent viruses. After periods of 2–7 days' infection feeding, the insects are little if any less infective during the first 24 hr. feeding on healthy plants than subsequently, the explanation being, of course, that the latent period is over before feeding on the healthy plants begins.

There is no clear evidence of any systematic change in the infectivity of the insects occurring after the end of the latent period. The infectivity appears to remain approximately constant over the whole period of the experiment.

The experiments have been arranged in order of increasing duration of infection feeding. It is not satisfactory, however, to accept these data, obtained with different batches of plants and insects, as showing how the infectivity of an aphis depends on the duration of the feeding time.

In Table 3 we set out in detail the results of one of the experiments summarized in Table 2. Each of the twenty aphides was tested successively on five healthy plants. There was therefore the possibility of obtaining 0, 1, 2, 3, 4, or 5 infections per aphis. Three of the twenty aphides gave no infections, six gave one infection, four gave two infections, two gave three infections, four gave four infections, and one gave five infections. In all there were forty-one infections obtained in 100 trials, i.e. an average of $p = 0{\cdot}41$ infection per trial. If we suppose that there is a certain probability p of obtaining an infection at one trial, and a probability $q = 1 - p$ of not obtaining infection, then according to the binomial distribution, the probability of obtaining 0, 1, 2, 3, 4, or 5 infections in five trials are respectively q^5, $5q^4p$, $10q^3p^2$, $10q^2p^3$, $5qp^4$, p^5. Inserting the values $p = 0{\cdot}41$, $q = 0{\cdot}59$, these probabilities are found to be 0·0715, 0·2484, 0·3452, 0·2399, 0·0834 and 0·0116. Multiplying by 20, the numbers of aphides expected to give 0, 1, 2, 3, 4, or 5 infections are deduced to be 1·4, 5·0, 6·9, 4·8, 1·7 and 0·2. The numbers experimentally obtained differ from these expected numbers in that more aphides than expected give large numbers (4 or 5) or small numbers (0 or 1) of infections, and fewer than expected give intermediate numbers (2 or 3). The difference is probably significant ($\chi^2 = 10{\cdot}0$, 3 degrees of freedom, $P = 0{\cdot}02$).

Tables 4 and 5 set out the results of two further experiments of the same sort. The discrepancy between the experimentally obtained frequencies and those calculated on the binomial distribution is in all cases in the sense that more aphides than expected produce *extreme* numbers of infections (many or few), and fewer than expected produce intermediate numbers of infections. The explanation is presumably that the aphides, though subjected as far as possible to similar conditions of infection feeding, are not all equally infectious. This explanation is confirmed by the fact that the

Table 2. *Proportions of infections obtained on successive healthy plants*

Date	Source	No. of aphides per plant	Duration of infection feeding	No. of plants per test	Proportion of plants infected in successive 24 hr. periods									
					1	2	3	4	5	6	7	8	9	10
June 1944	*R*	10	10 min.	12	0·17†	0·58†	0·33†	.	.	.	.	.	.	.
Apr. 1944	*V*	10	1 hr.	6	0·00	0·17	0·33	0·67*	0·83	0·17	.	.	.	.
Jan. 1939	*R*	1	24 hr.	6	0·17	0·33	0·50*	0·67	0·50	0·60	.	.	.	.
Sept. 1943	*R*	1	24 hr.	14	0·21	0·00	0·29*	0·29	0·36	0·29	.	.	.	.
Oct. 1943	*R*	1	24 hr.	20	0·05	0·50	0·60	0·45	.	.	.	.	.	.
Apr. 1944	*V*	1	24 hr.	12	0·08	0·08	0·50*	0·25	0·25	.	.	.	.	.
Dec. 1938	*R*	1	2 days	6	0·67	0·33	0·67*	0·00	0·33	0·50	.	.	.	.
Dec. 1938	*R*	1	3 days	5	0·2	0·2	0·6*	0·8	0·6	0·8	0·6	0·6	0·4*	0·6
June 1944	*R*	1	3 days	20	0·35	0·15	0·60*	0·45	0·50	.	.	.	.	.
Nov. 1943	*R*	1	5 days	20	0·95	0·90	0·90	0·90	.	.	.	.	.	.
June 1944	*V*	1	7 days	6	0·33	0·50	0·17*	0·17	0·17	.	.	.	.	.

* Feeding time on healthy plant 48 hr. instead of 24 hr.
† Feeding time on healthy plant 5 days instead of 24 hr.
R = source plant infected with the complex disease, rosette.
V = source plant infected with the vein-distorting virus only.

Table 3. *Numbers of plants infected by an aphis in five trials*

June 1944; rosette complex; 3 days' infection feeding.

	Successive transfers					
Aphis	1	2	3*	4	5	Totals
1	−	−	+	−	−	1
2	−	−	+	−	−	1
3	+	−	+	−	+	3
4	−	−	−	−	+	1
5	+	−	−	+	+	3
6	−	−	+	−	+	2
7	−	−	−	−	−	0
8	−	+	+	+	+	4
9	−	−	−	−	−	0
10	+	+	+	+	+	5
11	−	+	+	+	+	4
12	+	−	+	+	+	4
13	+	−	−	+	−	2
14	+	−	−	−	−	1
15	−	−	−	−	−	0
16	−	−	−	+	−	1
17	−	−	+	−	−	1
18	−	−	+	+	−	2
19	+	−	+	+	+	4
20	−	−	+	−	+	2
Totals	7	3	12	9	10	41

* 48 hr. transfer.

$$p = \frac{41}{20 \times 5} = 0{\cdot}41;\ q = 0{\cdot}59.$$

Infections per aphis	No. of aphides: Expected	Actual
0	1·4	3
1	5·0	6
2	6·9	4
3	4·8	2
4 or 5	1·9	5
Total	20·0	20

$\chi^2 = 10{\cdot}0$ for 3 degrees of freedom. $P = 0{\cdot}02$.

Table 4. *Numbers of plants infected by an aphis in four trials*

September 1943; rosette complex; 24 hr. infection feeding. First two trials omitted, being in the latent period.

	Successive transfers				
Aphis	3*	4	5	6	Totals
1	+	−	+	−	2
2	−	−	−	+	1
3	−	−	−	−	0
4	−	+	−	+	2
5	−	−	+	−	1
6	+	+	+	−	3
9	−	−	−	−	0
11	−	−	−	−	0
12	−	−	−	−	0
14	+	+	+	+	4
15	+	−	−	−	1
16	−	−	−	−	0
18	−	−	−	−	0
19	−	+	+	+	3
Totals	4	4	5	4	17

* 48 hr. transfer.

$$p = \frac{17}{14 \times 4} = 0{\cdot}3036;\ q = 0{\cdot}6964.$$

Infections per aphis	No. of aphides: Expected	Actual
0	3·3	6
1	5·7	3
2	3·8	2
3 or 4	1·2	3
Total	14·0	14

$\chi^2 = 7{\cdot}0$ for 2 degrees of freedom. $P = 0{\cdot}03$.

aphides which infect on the first day after the end of the latent period produce more infections per trial per aphis in subsequent trials than the aphides which do not infect on the first day after the latent period.

PART II. A QUANTITATIVE INTERPRETATION OF EXPERIMENTS ON THE TRANSMISSION OF VIRUSES BY APHIDES

1. *Introduction*

Discussions of the transmission of viruses by insects have usually (e.g. Storey, 1939; Bawden, 1943) drawn a sharp distinction between *persistent* and *non-persistent* viruses. The experiments of Watson (1936, 1938, 1940) and of Watson & Roberts (1939, 1940), however, have suggested that the difference in behaviour of the persistent virus, sugar-beet yellows, and the non-persistent *Hyoscyamus Virus* 1 is quantitative rather than qualitative. It is the purpose of the analysis which follows, which is mainly based on Watson's experiments, to reinforce this conclusion by showing that the principal features of the experiments on both viruses can be explained in a quantitative manner without invoking any assumptions which are *qualitatively* different for the persistent and non-persistent viruses. The differences in behaviour can be explained by a quantitative difference in the rates at which the viruses are inactivated while in the insect vector. This rate is rapid in the case of *Hyoscyamus Virus* 1 (reduced to half-activity in about 12 min.), slower in the case of sugar-beet yellows virus (reduced to half-activity in about 100 min.), and still slower in the case of rosette and mottle viruses (no reduction detectable in several days).

Watson's experiments on the manner in which the proportion of healthy plants infected depends upon the number of infective aphides feeding on each plant led to the following numerical result: If $1-q$ is the proportion of plants infected using 1 aphis per plant, and $1-Q$ is the proportion of plants infected using x aphides per plant, then $Q=q^x$. This is the relation to be expected if the aphides produce independent infections. She therefore concludes that when a plant on which several infective aphides has fed becomes infected, the infection is not to be ascribed to the cumulative effect of individually inadequate doses, but that at least one of the aphides gave a dose which would have caused infection even in the absence of the other insects.

In experiments on the transmission of viruses by single aphides it is found in general that of a number of aphides treated in an apparently identical manner (as regards feeding time on infected and healthy plants), some cause infection and some do not. It is not believed that this means that infecting and non-infecting strains are concerned in this experiment (as in Storey's experiments on the transmission of

Table 5. *Numbers of plants infected by an aphis in three trials*

October 1943; rosette complex; 24 hr. infection feeding. First trial omitted, being in latent period.

	Successive transfers			
Aphis	2	3	4	Totals
1	−	+	+	2
2	+	+	+	3
3	+	+	−	2
4	+	+	−	2
5	−	+	−	1
6	−	−	+	1
7	+	−	+	2
8	−	−	−	0
9	−	−	+	1
10	−	−	−	0
11	−	−	−	0
12	+	+	+	3
13	+	+	−	2
14	−	−	−	0
15	+	+	−	2
16	+	+	−	2
17	−	+	+	2
18	−	−	−	0
19	+	+	+	3
20	+	+	+	3
Total	10	12	9	31

$$p=\frac{31}{20\times 3}=0{\cdot}5167;\ q=0{\cdot}4833.$$

Infections per aphis	No. of aphides: Expected	Actual
0	2·2	5
1	7·2	3
2	7·8	8
3	2·8	4
Total	20·0	20

$\chi^2=6{\cdot}4$ for 2 degrees of freedom. $P=0{\cdot}04$.

maize streak by *Cicadulina mbila*). For the proportion of insects which transmit varies over a wide range, and in a fairly reproducible manner, with changes in the conditions of the experiment (more especially feeding and fasting times). It is presumed therefore that the proportion of plants infected is related to the mean dose of virus received per plant.

2. *Virus dosage*

We shall develop the subsequent analysis on the two basic postulates discussed above, namely:

(*a*) When several aphides are used per healthy plant, the aphides infect independently.

(*b*) In an experiment in which a batch of healthy plants receive treatments intended to be identical, the proportion of plants which are infected is related to the mean dose of virus received per plant.

It is convenient to be able to discuss the dose received by a plant in quantitative terms. As long as the proportion of infections is small, this proportion itself may be used as a measure of the mean dose of virus received per plant. Thus in experiments on the transmission of cucumber virus 1 by *Myzus persicae* using varying numbers of aphides per plant, and in which the proportion of infections obtained was below 0·3 even with the largest numbers of aphides per plant, it was found (Watson & Roberts, 1939, Table IV) that the proportion of plants infected was approximately proportional to the number of aphides per plant, i.e. to the dose received per plant. Thus as long as the proportion of infections is small, $1-q$ may be used as a measure of the mean dose of virus received per plant. However, if the proportion of infections is high, e.g. greater than 0·5, then $1-q$ is no longer a satisfactory measure of the dosage, since $1-q$ cannot exceed unity (when all the plants are infected) while there is in principle no limit to the dose which can be given per plant.

Table 6

No. of aphides per plant	1	5	10	20
Proportion of plants infected $= 1-q$	0·01	0·07	0·17	0·29

Instead, therefore, of using $1-q$ as a measure of the mean dose received per plant in an experiment in which a fraction $1-q$ of the plants are infected, we propose to use the quantity $m = \log_e (1/q)$. This tends to the limit $1-q$ when $1-q$ is small, but exceeds $1-q$ when $1-q$ is not small and is not subject to the upper limit of unity. It can readily be shown that the quantity m defined in this way has the correct properties justifying its use as a measure of the virus dose. If $1-q$ is the proportion of plants infected in an experiment using 1 aphis per plant, and $1-Q$ is the proportion infected in an experiment using x aphides per plant, then the corresponding doses are $m = \log_e (1/q)$ and $M = \log_e (1/Q)$, so that $q = e^{-m}$ and $Q = e^{-M}$. But the relation between Q and q expected on the assumption of independent infections, and experimentally verified, is that $Q = q^x$, whence $e^{-M} = e^{-mx}$, so that $M = mx$. In other words, the mean dose (M) per plant using x aphides is x times as great as the mean dose per plant (m) using 1 aphis per plant, showing that the quantity m has the correct properties.

The interpretation of experimental results in terms of mean dose per plant calculated in this way is illustrated in Table 7 by analysing an experiment on the variation of the proportion of infections with the number of aphides per plant (Watson, 1936, Tables VII and V). A reasonably consistent estimate of the dose delivered per aphis is obtained from the experiments using very different numbers of aphides per plant.

3. *Consecutive feeding experiments*

An infective aphis which is kept a long time (e.g. 24 hr.) on a healthy plant is not able to infect another plant on to which it is subsequently placed. If, however, an infective aphis is placed for a short time (e.g. 2–15 min.) on plant *A*, and then transferred to plant *B*, it sometimes happens that both plants are infected.

If, in an experiment of this sort, such as has been carried out on *Hyoscyamus Virus* 1 by Watson (1936), we calculate from the proportion of *A* plants infected the mean dose given per *A* plant, and from the proportion of *B* plants infected the mean dose given per *B* plant, then the *sum* of the doses per *A* plant and per *B* plant should be constant and independent of the duration of the feeding on the *A* plant (providing that the combined time on the two plants is sufficient to render all the aphides non-infective on subsequent plants). We show in Table 8 the results of Watson's experiments (Watson, 1936, Table XI) worked up in this fashion. In the last column the sum of the doses of virus given to the *A* and *B* plants is evaluated. The sums (for a given time on the source of infection) do not appear to show any systematic dependence upon the manner in which the time on the healthy plants is divided between *A* and *B* plants. (The large random fluctuations are inevitable in view of the small numbers of infections from which the estimates of dose are derived.)

The dose of virus which the aphis is able to give to the *B* plant diminishes rapidly with increase of the feeding time on the *A* plant. We test first whether the rate of decrease is different in the six series which differed in the lengths of time the insects spent on the source of infection. Table 9

Table 7A

No. of aphides per plant ...	1	5	10	20
Proportion of plants infected $(1-q)$	0·18	0·60	0·81	0·94
Dose (defined as $\log_e(1/q)$)	0·198	0·916	1·661	2·813
Hence dose delivered per aphis	0·198	0·183	0·166	0·141

Table 7B

No. of aphides per plant ...	1	5	10	20
Proportion of plants infected $(1-q)$	0·117	0·529	0·679	0·792
Dose (defined as $\log_e(1/q)$)	0·124	0·753	1·136	1·570
Hence dose delivered per aphis	0·124	0·151	0·114	0·079

Table 8. *Consecutive feeding*

Watson's 1936 experiments on *Hyoscyamus Virus* 1.

Time on source of infection	Time on first healthy plant	Proportion of infections		Dose given to		
		A	*B*	*A*	*B*	*A*+*B*
2 min.	2 min.	0·567	0·367	0·836	0·457	1·293
	5 min.	0·667	0·267	1·099	0·310	1·409
	15 min.	0·433	0·133	0·568	0·143	0·711
	1 hr.	0·600	0·000	0·916	0·000	0·916
	6 hr.	0·533	0·033	0·762	0·034	0·796
	12 hr.	0·767	0·000	1·455	0·000	1·455
5 min.	2 min.	0·367	0·233	0·457	0·266	0·723
	5 min.	0·433	0·333	0·568	0·405	0·973
	15 min.	0·333	0·000	0·405	0·000	0·405
	1 hr.	0·467	0·000	0·629	0·000	0·629
	6 hr.	0·600	0·000	0·916	0·000	0·916
	12 hr.	0·500	0·000	0·693	0·000	0·693
15 min.	2 min.	0·333	0·133	0·405	0·143	0·548
	5 min.	0·200	0·100	0·223	0·105	0·328
	15 min.	0·333	0·067	0·405	0·069	0·474
	1 hr.	0·300	0·000	0·357	0·000	0·357
	6 hr.	0·300	0·000	0·357	0·000	0·357
	12 hr.	0·600	0·000	0·916	0·000	0·916
1 hr.	2 min.	0·100	0·100	0·105	0·105	0·210
	5 min.	0·100	0·067	0·105	0·069	0·174
	15 min.	0·067	0·000	0·069	0·000	0·069
	1 hr.	0·067	0·000	0·069	0·000	0·069
	6 hr.	0·233	0·000	0·266	0·000	0·266
	12 hr.	0·133	0·000	0·143	0·000	0·143
6 hr.	2 min.	0·100	0·100	0·105	0·105	0·210
	5 min.	0·033	0·000	0·034	0·000	0·034
	15 min.	0·233	0·067	0·266	0·069	0·335
	1 hr.	0·200	0·067	0·223	0·069	0·292
	6 hr.	0·200	0·000	0·223	0·000	0·223
	12 hr.	0·400	0·000	0·511	0·000	0·511
12 hr.	2 min.	0·200	0·133	0·223	0·143	0·366
	5 min.	0·300	0·133	0·357	0·143	0·500
	15 min.	0·267	0·100	0·310	0·105	0·415
	1 hr.	0·067	0·000	0·069	0·000	0·069
	6 hr.	0·133	0·000	0·143	0·000	0·143
	12 hr.	0·300	0·000	0·357	0·000	0·357

Table 9

Infection feeding time ...	2 min.	5 min.	15 min.	1 hr.	6 hr.	12 hr.
Ratio of doses to *B* plants with 5 and 2 min. on *A* plants respectively	0·678	1·522	0·734	0·657	0·000	1·000

shows the ratio of the doses given to the *B* plants after 5 min. and after 2 min. feeding on the *A* plants respectively. Since there appears to be no very obvious systematic change of the rate of diminution of activity when different times of infection feeding are used, we average the data for all times of infection feeding to obtain a single curve of the diminution of dose given to the *B* plant with increase of the feeding time on the *A* plant. This curve is given in Fig. 1.

The smooth curve which has been drawn in Fig. 1, and which appears to be compatible with the experimental points, is an exponential curve, i.e. a curve indicating a constant rate of diminution of dose, in this case reduction of the dose to half in each period of 12 min. on the *A* plant. This curve may be represented by the formula: Fraction of virus dose remaining in insect after time t hr. is $e^{-\alpha t}$, where $\alpha = 3{\cdot}5$ hr.$^{-1}$.

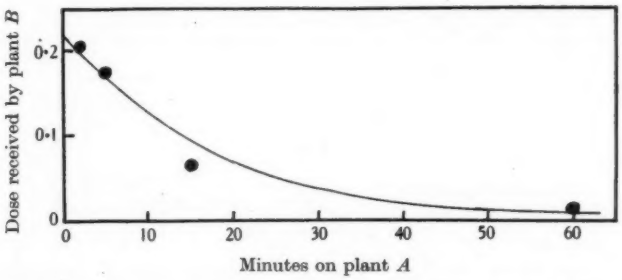

Fig. 1. Consecutive feeding experiment, *Hyoscyamus Virus* 1. (The curve is the exponential $e^{-\alpha t}$ with $\alpha = 3{\cdot}5$ hr.$^{-1}$. The points are derived from experiments of Watson (1936).)

Table 10. *Diminution of dose during post-infection fasting*

Watson's 1938 experiments on *Hyoscyamus Virus* 1

Duration of fasting ...		0	15 min.	1 hr.	6 hr.	12 hr.
Proportion of infections $= 1-q$	Exp. II	0·66	0·50	0·25	0·11	0·017
	Exp. III	0·64	0·44	0·18	0·04	—
	Exp. IV	0·66	0·35	0·28	—	0·033
Inferred virus dose $= \log_e (1/q)$	Exp. II	1·079	0·693	0·288	0·117	0·017
	Exp. III	1·022	0·580	0·198	0·041	—
	Exp. IV	1·079	0·431	0·329	—	0·034
	Mean dose	1·060	0·568	0·272	0·079	0·026

4. *Post-infection fasting*

Watson found that the number of infections obtained with *Hyoscyamus Virus* 1 diminished if a period of fasting intervened between the infection feeding and the infection trial. It is of interest to see how the rate of diminution during fasting compares with the rate of diminution during feeding on the *A* plants. Table 10 shows data obtained by Watson (Watson, 1938, Table VI) in three experiments, and in Fig. 2 the dose remaining after post-infection fasting is plotted against the duration of this fasting. The smooth curve is the same exponential curve which fitted the results of the consecutive feeding experiment. It is seen that for fasting periods up to about 15 min. the diminution occurs at about the same rate as in the consecutive feeding experiment, but that the rate subsequently diminishes. The explanation is probably that put forward by Watson (1938), namely, that the presence of digestive enzymes inactivates the virus in the insect, and the secretion of digestive enzymes ceases during starvation.

5. *Duration of feeding on the healthy plant*

Since the quantity of *Hyoscyamus Virus* 1 in an aphis diminishes rapidly after removal from the source of infection, and is reduced to a small fraction of the initial quantity after 1 hr., we should not expect the proportion of healthy plants which are infected to be increased greatly by feeding times on the healthy plants of more than 1 hr. The calculation can be made quantitative by supposing that the rate at which the aphis injects virus into the healthy plant is proportional to the quantity of virus in the

aphis at the particular moment, i.e. to $e^{-\alpha t}$. The total dose given to the healthy plant in time t can readily be shown to be proportional to $(1-e^{-\alpha t})$. Using the value $\alpha = 3{\cdot}5$ hr.$^{-1}$ deduced from the consecutive feeding experiment (p. 31), the manner in which the dose of virus given to the plant, and thence the proportion of infections, varies with the feeding time, is calculated and compared with the experimental values (Watson, 1936, Table IX, 1 aphis per

fection feeding following the preliminary fast. Accepting that (active) virus diminishes rapidly in the insect, the diminution of virus dose in the insect with increased duration of infection feeding can be explained in the following manner. An insect which has fasted for some time prior to the infection feeding presumably feeds much more rapidly than one which has not fasted, and the stomach therefore becomes filled with infective material before there

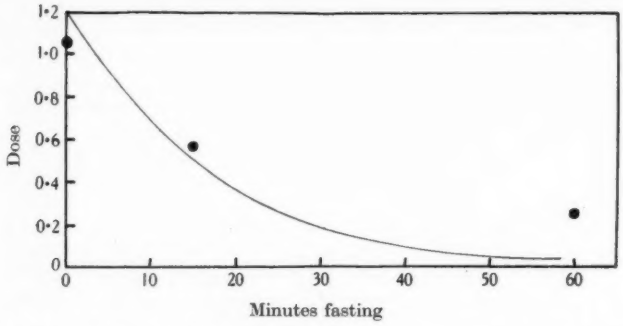

Fig. 2. Diminution of virus during post-infection fasting, *Hyoscyamus Virus* 1. (The curve is the exponential $e^{-\alpha t}$ with $\alpha = 3{\cdot}5$ hr.$^{-1}$. The points are derived from the experiments of Watson (1938).)

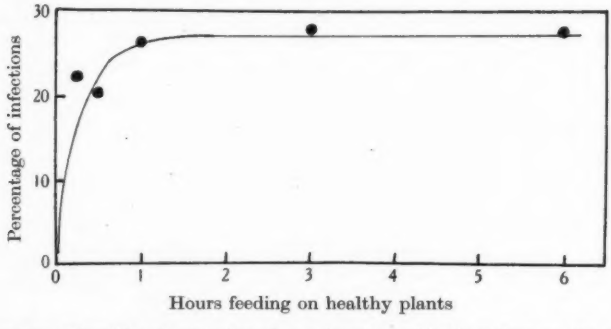

Fig. 3. Proportion of healthy plants infected as a function of feeding time on the healthy plants, *Hyoscyamus Virus* 1. (Curve theoretical, points experiments of Watson (1936).)

plant) in Fig. 3. The continuous curve in Fig. 3 shows the calculated values, and the individual points are the experimental values. Experimental and calculated values have been arranged to agree at 1 hr.

6. *Preliminary fasting*

Watson found that the proportion of healthy plants infected with *Hyoscyamus Virus* 1 was considerably increased if the aphides were made to fast for some time prior to a short feeding on the source of infection. The proportion of infections obtained was diminished by lengthening the duration of in-

is time for the virus activity to be destroyed. Such an insect, which has fed on the source just long enough for the stomach to be filled, will carry a maximum dose of virus. An insect which has been feeding on the source for a prolonged time is presumably feeding at a slower rate, and some of the stomach contents at any given moment were sucked from the source plant such a long time ago that the virus activity in them has seriously diminished. The dose of virus in the insect is thus less under these circumstances.

To make quantitative deductions requires a knowledge of the rate at which a previously starved aphis

takes up food when put on to the source of infection, compared to the average rate at which it takes up food when it has been on the plant for a prolonged time. This information could be obtained by weighing experiments. To illustrate how such information would be used, we make the following guesses, which will need modification if experimental data become available:

(*a*) Even 2 min. feeding of a previously fasting aphis suffices nearly to fill the stomach.

(*b*) The rate of feeding of an aphis after a prolonged time on a plant settles down to an average rate of one stomach-full per 2 hr.

If D is the dose of virus in one stomach-full of food, fully infective as taken from the source plant, then the dose carried by a starved aphis after a short feeding on the source is D. The dose carried by an aphis after prolonged feeding at the steady rate $\frac{1}{2}D$ per hour is readily shown to be $D/2\alpha$. An insect which after preliminary starving is made to feed on the healthy plant for some intermediate time t will have taken in dose D when first put on to the source, and subsequently will have taken in virus at the rate $\frac{1}{2}D$ per hour. The dose remaining at the end of this feeding period will be

$$D\left\{e^{-\alpha t}+\frac{1}{2\alpha}(1-e^{-\alpha t})\right\},$$

i.e.

$$D/2\alpha+D\,(1-1/2\alpha)\,e^{-\alpha t}.$$

In computing the theoretical curve of Fig. 4 we again take $\alpha = 3{\cdot}5$ hr.$^{-1}$, based on the consecutive feeding experiments (p. 31). The experimental values are those of Watson (1936, fig. 6), and experimental points and theoretical curve are arranged to agree for the shortest time of feeding (2 min.).

Watson (1938) has studied the effect of varying periods of preliminary fasting on the infectivity acquired by an aphis at a subsequent 2 min. infection feeding. The results expected in an experiment of this sort can be predicted: it is necessary as before to have information about the rate of feeding of an insect after a period of fasting, and in the absence of experimental data we shall need to make guesses. As before, we shall suppose that an insect which has fasted and has an empty, or partially empty, stomach is able to fill it after even a short time (2 min.) feeding on the source of infection. Also, we shall suppose that an insect which has fasted for 2 hr. or more has an empty stomach, one which has fasted for 1 hr. has a half-full stomach, one which has fasted for $\frac{1}{2}$ hr. has a three-quarters full stomach, and so on. These assumptions are consistent with the assumption previously made ((*b*), above) that the normal rate of feeding is one stomach-full per 2 hr., and are similarly subject to correction in the event of experimental data becoming available.

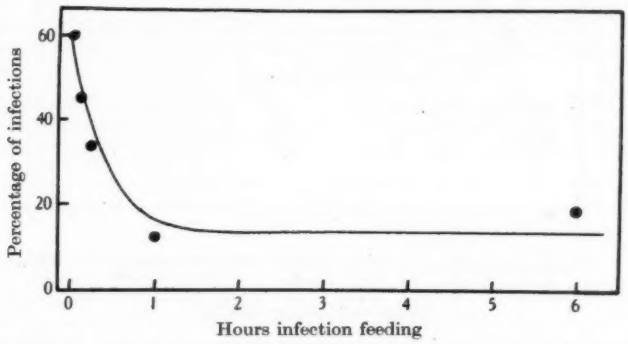

Fig. 4. Effect of duration of infection feeding, *Hyoscyamus Virus* 1. (Curve theoretical, points experiments of Watson (1936).)

It follows from these assumptions that the dose of virus taken in by an insect at a short (2 min.) infection feeding following a preliminary fast of t hr. attains a limiting (maximum) value for t greater than 2 hr., and for times t less than 2 hr. is a fraction $\frac{1}{2}t$ of this limiting value. Fig. 5 shows the calculated curve for comparison with the experimental results (Watson, 1938, Table III (solid dots) and Table IV (crosses). The data used are those for 2 min. infection feeding with no post-infection fasting).

7. *Conclusions regarding* Hyoscyamus Virus 1

It appears that a fair representation of the main experimental facts on the aphis transmission of *Hyoscyamus Virus* 1 can be obtained on the simple concept of diminution of virus activity at the rate $e^{-\alpha t}$, with $\alpha = 3{\cdot}5$ hr.$^{-1}$ (corresponding to reduction to half-activity after 12 min.).

In fitting theoretical formulae to experimental results principal weight has been attached to the shorter times (of infection feeding, fasting, etc.). The direction of the discrepancies between theory and experiment when longer times are taken into consideration suggests that the exponential function $e^{-\alpha t}$, while satisfactorily representing the facts for times up to about 1 hr., diminishes to zero too rapidly for times longer than 1 hr. At present it hardly seems worth while to use a more complicated mathematical function in the hope of getting a more exact fit.

In Watson's principal series of experiments (Watson, 1940, Tables 2 and 4) it was found that prolonged feeding on the source of infection, followed by prolonged feeding on the healthy plant, resulted in a fraction $1-q=0{\cdot}82$ of the plants becoming infected. Calculating the dose as usual it is $\log_e(1/q)=1{\cdot}72$. Reduction of the feeding time on the source of infection, or on the healthy plants, or on both, reduced the proportion of infections obtained.

Assuming that virus is taken in at a constant rate during infection feeding, but that its activity decays

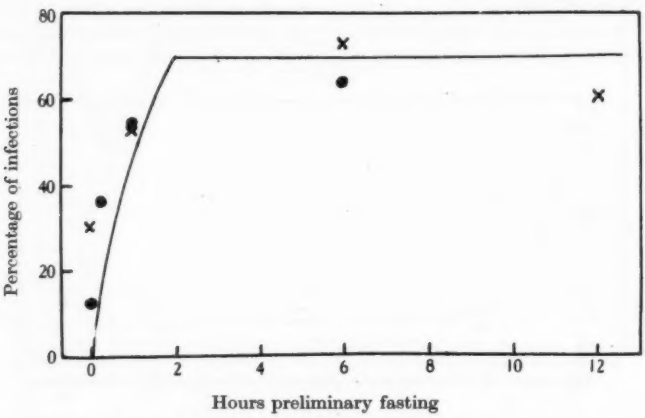

Fig. 5. Effect of duration of preliminary fasting, *Hyoscyamus Virus* 1. (Curve theoretical, points experiments of Watson (1938).)

8. *Watson's experiments on sugar-beet yellows virus*

These experiments deal with a 'persistent' virus. We shall see how far they can be explained along the same lines as were used in the case of the 'non-persistent' *Hyoscyamus Virus* 1. The rate of destruction of the activity of the virus in the insect is, of course, much slower than in the case of the non-persistent virus. We shall assume no qualitative difference, merely a quantitative difference in the value of α, which describes the rate of diminution of activity in the insect.

In one respect the calculations are simpler than in the case of the non-persistent viruses. Since very short feeding times (e.g. 2 min.) are not used, it is not necessary to take into account the rapid initial rate of feeding of a previously starved aphis when first placed on a plant, and it is sufficient to consider the feeding rate to be constant throughout the experiment.

Five aphides were used per healthy plant throughout Watson's experiments, and in the analysis the doses stated are always for five aphides.

in the insect according to the formula $e^{-\alpha t}$, then the dose of (active) virus in the insect after feeding for a time t is proportional to $(1-e^{-\alpha t})$.

When the infective insect is put on to the healthy plant, the activity of the virus in the insect diminishes according to the formula $e^{-\alpha t}$. Assuming that the rate at which virus is given to the healthy plant is proportional to the dose of virus in the insect at the given moment, it follows that the dose of virus given to the healthy plant in time T is proportional to $(1-e^{-\alpha T})$.

Combining the two formulae it follows that after a feeding time t on the source of infection and T on the healthy plant, the dose given to the healthy plant is proportional to $(1-e^{-\alpha t})\,(1-e^{-\alpha T})$. Making use of the result already mentioned that this dose has the value 1·72 when t and T are both long, we deduce that the dose after times t and T is

$$m=1{\cdot}72\,(1-e^{-\alpha t})\,(1-e^{-\alpha T}).$$

From the dose m the proportion of infections $1-q$ is deduced from the formula $q=e^{-m}$.

By trial and error the value $\alpha=0{\cdot}4$ hr.$^{-1}$ was

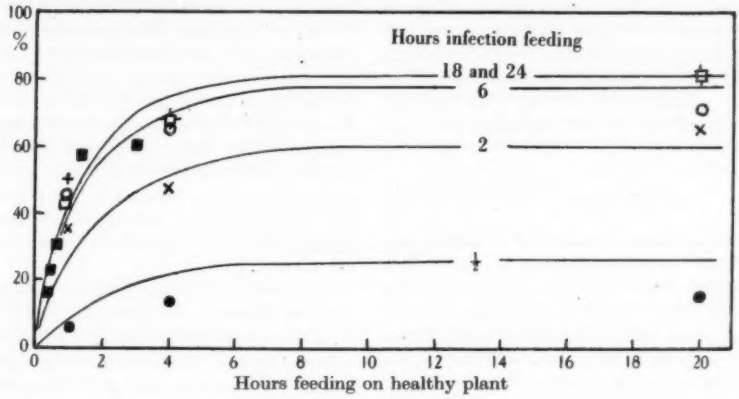

Fig. 6. Percentage of infections obtained with various periods on source of infection and on healthy plant. Sugar-beet yellows virus. (Curves theoretical, points experiments of Watson (1940).)

Experimental points:

● ½ hr. infection feeding, Table 2.
× 2 hr. infection feeding, Table 2.
○ 6 hr. infection feeding, Table 2.
□ 18 hr. infection feeding, Table 2.
\+ 24 hr. infection feeding, Table 2.
■ 18 hr. infection feeding, Table 1.

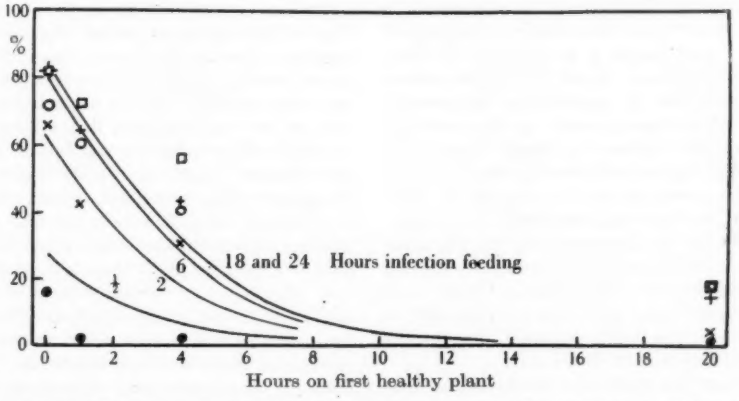

Fig. 7. Percentage of infections obtained in 20 hr. feeding on a second healthy plant after various periods of feeding on the source, and on a first healthy plant. Sugar-beet yellows virus. (Curves theoretical, points experiments of Watson (1940).)

Experimental points:

● ½ hr. infection feeding.
× 2 hr. infection feeding.
○ 6 hr. infection feeding.
□ 18 hr. infection feeding.
\+ 24 hr. infection feeding.

found most nearly to fit the results given in Watson's Table 2. The comparison between the theoretical curves and the experimental points is made in Fig. 6.

It is readily seen that after feeding for time t on the source of infection, for time T_1 on a first healthy plant, and for time T_2 on a second healthy plant, the dose given to the second healthy plant will be $1{\cdot}72\,(1-e^{-\alpha t})\,e^{-\alpha T_1}\,(1-e^{-\alpha T_2})$. Using the same value of α the doses, and hence the proportion of infections to be expected in the second healthy plant, have been calculated for $T_2 = 20$ hr. and for various values of t and T_1. The theoretical values are compared with the experimental results (Watson, 1940, Tables 2 and 4) in Fig. 7, in which the proportion of infections on the second plant is shown as a function of

the feeding time on the first healthy plant. There is moderate agreement for times up to 4 hr. on the first healthy plant, but it is evident that for longer times the virus activity does not actually fall off so rapidly as the exponential function. This was also noted in the analysis of the experiments with *Hyoscyamus Virus* 1.

DISCUSSION

It appears that the main features of Watson's experimental results on *Hyoscyamus Virus* 1 and sugar-beet yellows virus can be explained on the basis that virus activity diminishes in the insect, rapidly in the former case, less rapidly in the latter case. For the purpose of numerical calculation we have assumed the rate of diminution of virus activity to be constant for a given virus, i.e. to follow the formula $e^{-\alpha t}$. From the direction of certain discrepancies which occur between experimental and theoretical results, it is evident that the rate of inactivation is actually not constant but becomes slower in experiments extending over a long time. It would be possible to improve the agreement between experiment and theory in some cases by assuming a more complicated relation between virus activity and time than the simple exponential. While we have not thought it worth while to introduce this complication, it is not difficult to understand why the rate of inactivation apparently diminishes. In the experiments on the vein-distorting and mottle viruses in which there is no appreciable inactivation in the insect, the infectivity of an insect increases during the first 48 hr. after the completion of a short period of infection feeding, this perhaps being the time required for the virus to reach its full concentration in the salivary glands. In the experiments with *Hyoscyamus Virus* 1 and sugar-beet yellows virus this slow increase will be superimposed on the rapid decrease due to the inactivation of the virus in the insect.

The view that the difference between the persistent and the non-persistent viruses is in the main a quantitative rather than a qualitative one affords an explanation of the fact that non-persistent viruses, in contrast to persistent viruses, are usually mechanically sap-transmissible. The argument is as follows: If two viruses were present in their respective hosts in equal concentrations and were equally virulent, but differed in that one was non-persistent (the activity falling off in the insect vector in a few minutes) and the other was persistent (the activity remaining in the insect vector for many hours), then the proportion of transmissions obtained with the two viruses using *short* feeding times on the source and healthy plants would be equal. With prolongation of the feeding times on source and healthy plant the percentage of transmission obtained with the non-persistent virus would not increase. The percentage of transmission obtained with the persistent virus would, however, increase (the *dose* given to the healthy plant being proportional to the product of the feeding times on source and healthy plant). Consequently with optimum feeding times on source and healthy plant (which means quite short times for the non-persistent, and long times for the persistent viruses) the proportion of infections obtained would be higher with the persistent than with the non-persistent virus.

Conversely, if instead of supposing our two hypothetical viruses to be present in equal concentration in their respective hosts we suppose that under optimum conditions they produce equal percentage transmission, it follows that the non-persistent virus must be present in the source plant in much higher concentration (or must be much more virulent) than the persistent virus.

Thus the non-persistent viruses are all viruses which are present in high concentration in their source plants (or are of high virulence). It is natural, therefore, that the non-persistent viruses should be usually mechanically sap-transmissible. Doubtless there are viruses present in low concentration in their source plants of which the activity falls off rapidly in the insect. Such viruses will be regarded as non-transmissible by the insect, as well as non-sap-transmissible. On the other hand, a virus present in low concentration in the source plant, but of which the activity did not fall off rapidly in the insect vector, might very well be insect-transmitted. It appears that, in general, insect transmission (of a persistent virus) is a more efficient way of transmitting virus from the source plant to the sensitive part of a healthy plant than is mechanical inoculation. Consequently viruses present in concentration too low to be sap-transmitted are often transmitted by insects. There is no *a priori* reason why a virus present in sufficient concentration in the source plant to be mechanically sap-transmissible should not retain its activity in the insect vector and so behave as a persistent virus. Thus we should expect some persistent viruses to be sap-transmissible, and this is in fact the case (e.g. the viruses of mottle, tomato spotted wilt, potato yellow dwarf, and pea virus 1).

The simple quantitative arguments we have put forward obviously leave many facts concerning insect transmission unexplained, e.g. the fact that tobacco mosaic virus is not insect-transmitted, and the specificity of the relation between viruses and their insect vectors, and for these separate explanations will be needed. It is, however, desirable that it should be realized that a number of differences in behaviour between different viruses can be explained as simply quantitative differences, namely,

differences in their concentration in their host plant (or in their virulence), and differences in the rates of inactivation in the insect vectors.

SUMMARY

1. Experiments are described on the transmission of mottle and vein-distorting viruses by aphides.

2. A quantitative analysis is made of Watson's experiments on the aphis transmission of persistent and non-persistent viruses. The principal features of the experimental results can be explained in terms of different rates of inactivation of the virus in the insect.

3. It is shown that these considerations explain why non-persistent viruses are more commonly sap-transmissible than persistent viruses.

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A RECORD OF THE NEMATODE PARASITES OF FISHES FROM THE PORCUPINE BANK, IRISH ATLANTIC SLOPE AND IRISH SEA

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The nematodes recorded in the present paper were obtained from fishes caught on some of the deep-sea fishing grounds to the west of Ireland, namely, the Porcupine Bank and Irish Atlantic Slope, and from the Irish Sea. The writer is indebted to Mr S. J. Hughes and Mr J. Llewellyn for the collection of material from the deep-sea fishing grounds, excursions having been made for the purpose in a commercial trawler in August 1938 and July 1939. The majority of the nematodes were collected by Mr S. J. Hughes during the 1939 excursion, the first excursion being devoted mainly to the collection of trematodes and cestodes. A record of the trematode and cestode parasites obtained during both years has already been published (Rees & Llewellyn, 1941). Nematodes from fishes in the Irish Sea have been obtained at intervals from 1936 onwards.

The trematodes, cestodes, nematodes and acanthocephala from fishes in rock pools near Aberystwyth have been listed more recently (Rees, 1945). The present paper therefore completes the investigation of the helminth material which has been available to the writer, during the last few years, from fishes off the west coast of this country.

Records of nematode parasites from fishes around these islands are rare. Baylis (1928, 1939*b*) published lists of parasites obtained from vertebrates 'within these islands and off their coasts', including therefore some nematodes from fishes. The lists are important as they contain only those worms actually found. Many of the previous lists, of trematodes more particularly, were based largely on continental records, some of the species in question never actually having been recorded for this country. Baylis & Jones (1933) included nematodes in their list of parasitic worms from marine fishes at Plymouth, but otherwise little attention has been paid to the nematodes of British fishes.

Records of fish nematodes have been made from time to time on the continent of Europe by Rudolphi (1809), von Linstow (1899), Dujardin (1845), Diesing (1851), Wülker (1930) and others.

The localities from which fishes were obtained for the present study are indicated in Table 1. The numbers given to the localities will be referred to again in Tables 2–4.

In all, 703 host fishes belonging to forty-seven different species were examined. Those caught by the trawler were investigated immediately and those from the Irish Sea were sent in to the laboratory at Aberystwyth. The nematodes were fixed in hot 70% alcohol and examined later in lactophenol. A list of the fishes in which nematodes were found is given in Table 2, together with their parasites and the localities from which they were obtained. The names used for the fishes are those in the *British Museum List of British Vertebrates*, 1935.

Table 1. *Localities from which fishes were obtained*

1.	52° 50′ N., 4° 05′ W.	Irish Sea (depth 0–50 fm.)
2.	53° 40′–54° N., 11° 40′ W.	Irish Atlantic Slope (depth 120–150 fm.)
3.	52° 25′–53° 35′ N., 12° W.	
4.	53° 40′–53° 45′ N., 12° W.	
5.	53° 45′–53° 55′ N., 14° W.	Porcupine Bank (depth 160–200 fm.)

Twenty-one species of fishes were found to be infested and these were distributed between localities 1, 2, 3 and 5. The host fish containing the largest number of nematode species was *Conger conger*, with seven. This may be accounted for by the fact that the conger is a migratory fish and is therefore more liable to be infested with a large number of different species than are fishes more restricted in their distribution. In Table 3 are included those fishes in which no nematode parasites were found. The localities and the number examined in each case are indicated.

Twenty-six species of fishes were found to be without nematode parasites. These were distributed amongst all the localities. Although there were more host-species without parasites than with them, the total number of fishes examined in Table 3 amounted to only 116 and in Table 2 to 587 (for details of numbers of infested species see Table 4). The fact that in the former case fewer individuals, on the whole, per species were examined may account for the apparent absence of infestation.

A classified list of the nematodes with their hosts and the numbers of hosts examined and found infested is given in Table 4. The classification adopted is that of Baylis (1936, 1939*a*).

Thirteen species of nematodes were recovered from the total of 703 fishes examined, a percentage infestation of almost 30.

The nematodes were distributed as follows. From

Table 2. *List of host fishes with their nematode parasites* (see Rees & Llewellyn, 1941)

Host	Locality	Parasite
Conger conger (L.)*	1 and 2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Capillaria* sp., intestine
,,	1 and 2	*Contracaecum clavatum*, stomach and intestine
,,	2	*Contracaecum* sp., intestine
,,	1 and 2	*Cucullanus hians*, intestine
,,	2	*Proleptus africanus*, intestine
,,	2	*Proleptus obtusus*, stomach
Gadus merlangus L.*†	1 and 2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	1 and 2	*Contracaecum clavatum*, stomach and intestine
G. virens L.*†	2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum clavatum*, stomach and intestine
Limanda limanda (L.)†	1	*Cucullanus* sp., intestine
Lophius piscatorius L.	2	*Anisakis* or *Porrocaecum* sp., peritoneum
Merluccius merluccius (L.)*†	2 and 5	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum* sp., intestine
Molva byrkelange Walb.	5	*Anisakis* or *Porrocaecum* sp., peritoneum
M. molva (L.)*	5	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	5	*Spinitectus cristatus*, intestine
Osmerus eperlanus L.	2 and 5	*Anisakis* or *Porrocaecum* sp., peritoneum
Pagellus centrodontus (De la Roche)*	3	*Anisakis* or *Porrocaecum* sp., peritoneum
Raja batis L.*†	2	*Proleptus robustus*, intestine
R. clavata L.†	1	*Proleptus robustus*, intestine
Salmo salar L.*†	1	*Anisakis* or *Porrocaecum* sp., larvae, intestine
S. trutta L.*†	1	*Cucullanus truttae*, intestine
Scomber scombrus L.*	2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum clavatum*, intestine
Serranus cabrilla (L.)†	5	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	5	*Contracaecum* sp., peritoneum
,,	5	*Camallanus* sp., intestine
Scyliorhinus caniculus (L.)†	1	*Proleptus obtusus*, stomach and intestine
Trachurus trachurus (L.)	2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum clavatum*, intestine
Trigla cuculus L.*	2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum* sp., peritoneum
T. gurnardus L.*	2	*Anisakis* or *Porrocaecum* sp., peritoneum
,,	2	*Contracaecum* sp., peritoneum
T. lyra L.	2 and 5	*Anisakis* or *Porrocaecum* sp., peritoneum

* Trematodes. † Cestodes.

Table 3. *List of fishes without nematode parasites* (see Rees & Llewellyn, 1941)

Host	Locality	No. examined	Host	Locality	No. examined
Arnoglossus megastoma Donovan	4	6	*Pleuronectes platessa* L.	1	32
Brosme brosme Müller	3	2	*Raja fullonica* L.*	5	4
Centrophorus squamosus (Gmelin)†	5	5	*R. montagui* Fowler*†	4	2
Chimaera monstrosa L.*†	2 and 4	3	*R. microcellata* Montague*	5	2
Clupea harengus L.	1	1	*R. naevus* Müller & Henle*†	5	12
Gadus aeglefinus L.	2	2	*R. oxyrhynchus* L.*	2	1
G. callarias L.	2	1	*R. undulata* Lacepède	5	3
G. pollachius L.	2	2	*Scyliorhinus stellaris* (L.)†	1	1
Glyptocephalus cynoglossus (L.)†	5	6	*Scymnorhinus licha* (Bonnaterre)*	5	4
Hexanchus griseus (Gmelin)*†	3 and 5	4	*Solea solea* (L.)	1	3
Lepidorhynchus whiff-iagonis (Walb.)	3	2	*Spinax spinax* (L.)*	5	6
Mustelus mustelus L.†‡	1	1	*Torpedo nobiliana* Bonaparte*	1	6
Platichthys flesus (L.)	1	3	*Urophycis blennoides* (Brunnich)*	3	2
Total 26 species			116 fishes		

* Trematodes. † Cestodes. ‡ *Anthobothrium cornucopia*—not recorded previously for this area.

Table 4. *List of nematodes with their hosts*

Nematode	Host	Locality	No. examined	No. infested
ASCAROIDEA				
ASCARIDAE				
Anisakinae				
1. *Anisakis* or *Porrocaecum* sp. (larva)	*Conger conger*	1 and 2	4	3
	Gadus merlangus	1 and 2	56	7
	G. virens	2	21	12
	Lophius piscatorius	2	9	9
	Merluccius merluccius	2 and 5	36	29
	Molva byrkelange	5	5	5
	M. molva	5	17	3
	Osmerus eperlanus	5	36	1
	Pagellus centrodontus	3	47	7
	Scomber scombrus	2	6	3
	Serranus cabrilla	5	5	5
	Trachurus trachurus	2	38	13
	Trigla cuculus	2	1	1
	T. gurnardus	2	7	6
	T. lyra	5	20	1
	Salmo salar (intestine)	1	1	1
2. *Contracaecum clavatum* (Rud.)	*Conger conger*	1 and 2	4	4
	Gadus merlangus	1 and 2	56	15
	G. virens	2	21	11
	Scomber scombrus	2	6	1
	**Trachurus trachurus*	2	38	3
3. *Contracaecum* sp.	*Conger conger*	2	4	1
	**Merluccius merluccius*	2	36	1
4. *Contracaecum* sp. (larva)	**Serranus cabrilla*	5	5	2
	**Trigla cuculus*	2	1	1
	**T. gurnardus*	2	7	3
FILARIOIDEA				
SPIRURIDAE				
Physalopterinae				
5. *Proleptus africanus* (v. Linst.)	**Conger conger*	2	4	1
6. *Proleptus obtusus* Duj.	**C. conger*	2	4	3
	Scyliorhinus caniculus	1	11	11
7. *Proleptus robustus* (v. Beneden)	**Raja batis*	2	11	4
	R. clavata	1	12	2
Thelaziinae				
8. *Spinitectus cristatus* Railliet & Henry	*Molva molva*	5	17	1
CAMALLANIDAE				
9. *Camallanus* sp.	**Serranus cabrilla*	5	5	1
CUCULLANIDAE				
10. *Cucullanus hians* (Duj.)	*Conger conger*	1 and 2	4	2
11. *Cucullanus truttae* Fabr.	*Salmo trutta*	1	2	1
12. *Cucullanus* sp.	*Limanda limanda*	1	26	1
TRICHINELLOIDEA				
TRICHINELLIDAE				
Trichurinae				
13. *Capillaria* sp.	**Conger conger*	2	4	1

the deep-sea fishing grounds only, six species, namely, *Contracaecum* sp., *Contracaecum* sp. larva, *Camallanus* sp., *Capillaria* sp., *Proleptus africanus* and *Spinitectus cristatus*; from the Irish Sea only, two species, namely, *Cucullanus truttae* and *Capillaria* sp.; and from both the deep-sea fishing grounds and the Irish Sea five species, *Anisakis* or *Porrocaecum* sp. larva, *Contracaecum clavatum*, *Cucullanus hians*, *Proleptus obtusus* and *P. robustus*.

In a few cases the nematodes could not be

specifically determined owing to the absence of males or to the immaturity of the specimens. In the cases of *Capillaria* sp. from *Conger conger* and *Cucullanus* sp. from *Limanda limanda*, one female only was found. There were several females of *Camallanus* sp. in *Serranus cabrilla*, but they were young.

The most widely distributed nematodes among the hosts were the larval ascarids, occurring in the peritoneum of fifteen different host species. Most of these were from the deep-sea fishing grounds only, except for *Conger conger* which was infested both there and in the Irish Sea, and *Gadus merlangus*, infested in the Irish Sea only. As the conger is migratory, one would expect the same parasites in both areas. Larval ascarids were also found in the intestine of *Salmo salar* in the Irish Sea. It is probable that these had been derived from some other fish recently swallowed. In the adult stage the worms may occur in the stomach and intestine of marine mammals, which perhaps accounts for their appearance in the Irish Sea, as porpoises and dolphins are seen there at times.

Whether or not all these larvae belong to the same species or even genus, is impossible to say. They all possess an elongated 'ventriculus' but have no intestinal caecum, a condition which characterizes the adults of the genus *Anisakis*. They may even be larvae of *Anisakis simplex* Duj., the adults of which occur in Cetacea. On the other hand, the same type of oesophagus may occur in the larvae of *Porrocaecum*, the adults of which also possess an intestinal caecum. But young larvae of *Porrocaecum* cannot be distinguished with certainty from those of *Anisakis*, as the intestinal caecum of the former develops late. The writer therefore adopts the suggestion made by Baylis (1929) that larval ascarids of this type should be referred to as *Anisakis* or *Porrocaecum* sp.

Various authors have claimed to be able to identify specifically larval stages of ascarids from marine fishes, but it is very doubtful if this is possible. Baylis (1944) has made some interesting observations on this question arising out of a paper by Johnston & Mawson on ascarid nematodes from Australian fishes.

Contracaecum clavatum occurred in five species of host fishes and again in both areas. *C. clavatum*, however, seems to be a very widely distributed species. It occurs in *Cottus bubalis* and *Onos mustelus* in rock pools at Aberystwyth, and Baylis & Jones (1933) have recorded it from *Conger conger*, *Gadus merlangus*, *Merluccius merluccius*, *Lophius piscatorius*, *Zeus faber*, *Cottus bubalis*, *Capra aper* and *Mullus surmuletus* at Plymouth.

The two species restricted to the Irish Sea, namely, *Cucullanus truttae* from *Salmo trutta* and *Cucullanus* sp. from *Limanda limanda*, occur in hosts not found on the deep-sea fishing grounds.

Of the species from the deep sea, only one female of *Contracaecum* sp. was obtained from *Conger conger* and one from *Merluccius merluccius*. A larval form of *Contracaecum* occurred in the peritoneum of three host fishes, but it was not possible to determine the species of this larva. An interesting record is that of *Proleptus africanus* (v. Linst.) from *Conger conger*. The determination of this species may be open to question, but the specimens agree very closely with the description given by von Linstow (1899) of *Spiropterina africana* from *Anguilla* sp. in South Africa. The species was placed in the genus *Proleptus* by Baylis (1933).

Hosts believed to be new for any of the species of nematodes found are indicated in Table 4 by an asterisk. Larval ascarids have been recorded as occurring in various fishes, but the names of the hosts are not always given, and it is impossible to know in all cases if they are the same as those found in the present investigation. None of these, therefore, is marked, though some of the host records may be new.

The writer expresses her gratitude to Major T. R. Ronald of Consolidated Fisheries Ltd., and to the skipper and crew of the S.T. *Lionheart*, whose valuable assistance made the collection of the deep-sea material possible.

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NOTES ON *TROMBICULA DELIENSIS* WALCH, 1923 (ACARINA; TROMBIDIIDAE), WITH DESCRIPTION OF THE ADULT*

By CHARLES D. RADFORD, *Squadron Leader, R.A.F. Acarologist*

(With 9 Figures in the Text)

INTRODUCTION

Life histories of the Trombidiidae, or, for that matter, other families of mites have, with few exceptions, not been studied and very little information is available as a guide. This is not entirely the fault of the acarologists, as not one of them was employed in any country of the world entirely upon the study of mites. Most of them, if official, were entomologists who could spend only a fraction of their time on mites. Their time was spent therefore on systematics, an essential branch of acarology.

Most of the early work upon the life histories of the Trombidiidae was carried out by the Japanese workers, Miyajima & Okumura, Kawamura & Yamaguchi, and Nagayo *et al.* Their reasons for studying the Trombidiid mites was the fact that Japanese river fever or 'tsutsugamushi disease' was present in certain areas of Japan, and it was suspected that the larva of *Trombicula akamushi* Brumpt, 1910 was the vector.

Miyajima & Okumura (1917) published an account of the life cycle of *T. akamushi*, and Nagayo, Miyagawa, Mitamura, Tamiya & Tenjin (1921) record the successful rearing of larvae of *T. akamushi* Brumpt, *T. pallida* Nagayo, 1919, *T. palpalis* Nagayo, 1919 from adults, and of obtaining the nymphs of the four species *T. akamushi*, *T. pallida*, *T. palpalis* and *T. scutellaris* Nagayo, 1920 from fully engorged larvae. They also state: 'so far we have succeeded in our breeding experiments in obtaining adults of *T. akamushi*, *pallida* and *palpalis*'.

From this work, carried out by the Japanese, it would appear that in the nymphal and adult stages these mites are plant feeders, living on the juice of reeds (*Imperata arundinacea* Cyr.) and a daisy (*Artemisia vulgaris* L.).

Walch (1923) published a description of the larval and nymphal stages of *Trombicula deliensis*, the suspected vector of scrub-typhus in Deli, Sumatra. Ewing records the successful rearing of *T. irritans* Riley, 1873 in America, and Hirst (1915) records the rearing of *T. autumnalis* Shaw, 1790 in England, as far as the nymphal stage.

Attempts to rear the mite *T. deliensis* Walch, 1923 have been conducted in Ceylon during 1944 (May to August), and though the experiments have not established the full life cycle, the technique and results may be of interest to acarologists working along similar lines. Herein is described and figured the adult which, until now, has been unknown.

TECHNIQUE

A number of small mammals and birds have been examined, and from them larvae of *T. deliensis* have been taken. Among the hosts examined and found to carry these larvae are the Ceylon house rat (*Rattus rattus kandianus* Kelaart), the bandicoot (*Bandicota malabarica* Shaw), the musk shrew (*Suncus caeruleus giganteus* Geoffroy), the palm squirrel (*Funambulus palmarum favonicus* Thomas & Wroughton), the gerbille (*Tatera indica ceylonica* Wroughton), the house crow (*Corvus splendens*), the koel (*Eudynamis scolopaceus* L.), also the crow pheasant (*Centropus sinensis* Stephens).

Often the larvae were found in clusters of from four to a dozen in the ears of the house rat (*Rattus r. kandianus*), but seldom found upon the body. The ears of the bandicoot (*Bandicota malabarica*) were found to be more heavily infested, and a cluster of 100–200 larvae in each ear was not uncommon. The musk shrew (*Suncus c. giganteus*) was found to have few larvae in the ears, which were coated in a blackish grease. It was observed that the mites were always found upon the hind thighs in the fur just above the bare leg.

A small quantity of thoroughly washed sand and crushed, dead leaves moistened with rain water was placed in 3 by 1 in. glass specimen tubes

Legends to figs. 1–9

Fig. 1. *Trombicula deliensis* Walch. Adult venter.
Fig. 2. *Trombicula deliensis* Walch. Adult dorsum.
Fig. 3. *Trombicula deliensis* Walch. *a*, dorsal setae; *b*, dorsal seta enlarged.
Fig. 4. *Trombicula deliensis* Walch. *a*, crista; *b*, sensory seta.
Fig. 5. *Trombicula deliensis* Walch. Tarsus i.
Fig. 6. *Trombicula deliensis* Walch. *a*, tarsus ii; *b*, tarsus iii; *c*, tarsus iv.
Fig. 7. *Trombicula deliensis* Walch. Mandible.
Fig. 8. *Trombicula deliensis* Walch. Palp.
Fig. 9. *Trombicula deliensis* Walch. Genital pore.

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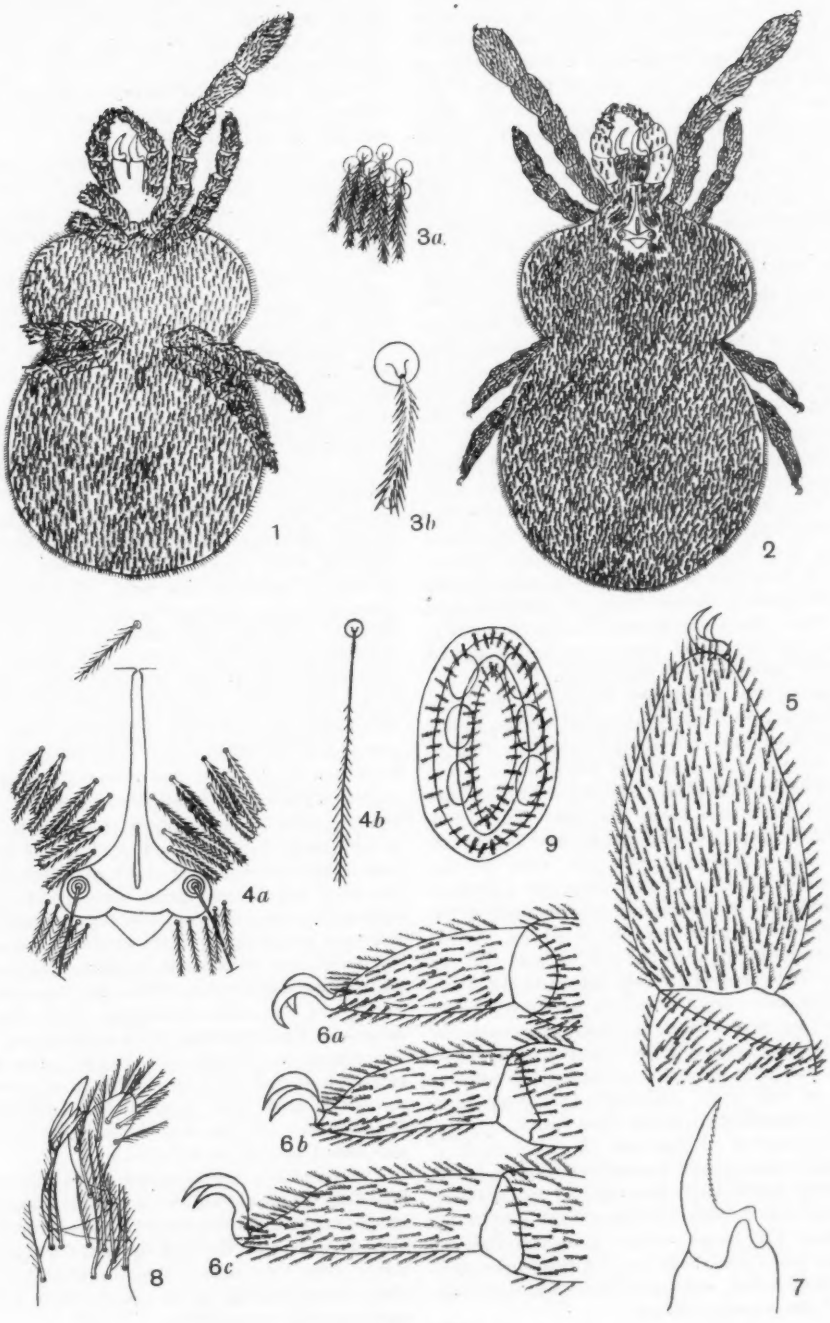

Figs. 1–9.

(corked). Larvae taken from the rats' ears were found either unengorged or in removing them were damaged. It was therefore considered more suitable to the success of the experiment to cut off the ear containing the mites and to trim off the bulk of the flesh, leaving only that portion of the ear upon which the mites were attached. This was then placed in the tube, and the day following the ear was removed, as most of the mites had left it and burrowed into the soil. Daily examinations of the tubes showed that for 48 hr. after being placed in the tube some mites could be found walking upon the glass, but few were visible after the second day. On the ninth day some of the nymphs could be seen walking about in the tube, and in some instances the larval skin was observed to be transparent, and the nymph could be seen inside. On a number of occasions the emergence of the nymph from the larval skin was observed. In one instance the nymphs did not appear until the sixteenth day.

The number of nymphs raised from some of these larvae was small, but in one case seventy-three nymphs were obtained from a tube into which 100 larvae had been placed. The nymphs remained active for 2 or 3 days but were all dead within 4 days. It has been stated that the nymphs and adults will feed upon the juice of potatoes and melons, but in these experiments it was found that the mites would approach the potato and either be poisoned by the juice or drowned in it.

Apparently the life-history studies which have been conducted by Hirst, Walch, McCulloch, Philip and Kohls have all had equal success (or rather, failure), the nymphs dying after the first few days.

As the life cycle could not be continued from this approach, search was made for the adult mites amongst the vegetation near the rat and bandicoot burrows, but without success. In view of the abundance of larvae on bandicoots the soil from the entrances to their burrows was examined for free-living mites, larvae, nymphs or adults. A few adults were found in the top 3 in. of soil, but no specimens were recovered from layers below 3 in. A more abundant supply of adults (of six genera of Trombidiidae) was found beneath a growth of coarse, low-lying grass, under the shade of trees. The trees and grass served to conserve the surface moisture of the soil. Although the grass was thoroughly examined no mites were found.

The examination of the soil (dry) in shallow photographic trays (white enamel) was both tedious and not very productive. The method found most suitable was the flooding of these trays with rain water, when the fauna—mites, pseudoscorpions, Coleoptera, and Collembola—would float upon the surface. Magnesium sulphate was tried but the results did not warrant its use.

Observations of the mites under a binocular microscope showed that the adults were easily destroyed when moving by sharp sand or wood splinters tearing open the body. This precluded the possibility of utilizing any of the soil sampling methods such as the Ladell or Evans system, in which a strong jet of water was used to break up the soil for examination.

One of the adult genera recovered from the soil was found to agree in specific characters with the nymphs reared in the laboratory, the identity of which had been confirmed by comparison with the figures given by Walch.

For facility in examination, staining cells (or glass blocks) $1\frac{1}{2}$ in. square (37 mm.) with a central cavity were found to be more suitable than the round 3 by 1 in. glass specimen tubes. The sand, leaf mould and rain water mixture as used in the rearing of nymphs was placed in these cells. The glass cell covers were attached with a thin smear of vaseline to prevent the mites escaping. During the first 48 hr. some of the adult mites would get between the rim of the cell and cover-glass, embedding themselves in the smear of vaseline. If observed soon enough they appeared to be none the worse for the vaseline when placed in the soil, but too heavy a coating of vaseline was fatal. Unfortunately the glass cells soon developed a growth of mould, in the threads of which the mites became trapped. To prevent this mould formation and to keep the cells ventilated a cover was made of $\frac{3}{16}$ in. aluminium, $1\frac{1}{2}$ in. square with a central hole $\frac{3}{4}$ in. in diameter, over which a square of bolting silk was gummed. This prevented the adult mites from escaping and was kept in position with a thin smear of vaseline. Small pieces of the coarse grass were placed in each cell, but the mites did not appear to make any attempt to feed upon it. Three or four drops of rain water were added to each cell night and morning as required. When the moisture of the soil had evaporated during the day, the mites would remain inactive, but with the addition of water they became active.

Though the cells were examined at least once daily, sometimes more often, no ova were found in any of the cells containing adult *Trombicula deliensis*. Nineteen days after preparation, one cell was found to contain two larvae. One of these larvae was lost but the other was successfully mounted, and upon examination proved to be *T. deliensis*. On the day following a second cell was found to contain two larvae.

The mortality amongst adult *T. deliensis* was found to be great, and it was not uncommon for specimens to die a few hours after sorting from the soil. If specimens floating upon the surface of the water were very active they invariably lived for many days, whilst those which were weak and inactive soon succumbed.

The supposition that these specimens were adults

was established by the finding in each of eight specimens of a single ovum and subsequently confirmed in many other mounted specimens. This species does not, in common with some genera of the Trombidiidae, produce batches of ova, and in several hundred specimens examined there has never been more than one ovum. In one adult a fully formed larva could be seen inside the ovum *in situ*.

Experiments to ascertain the influence of varying conditions of soil, humidity, etc., were tried—soil from normal habitat kept without addition of moisture, dry sand, leaf mould, moist sand and leaf mould, moist filter paper, etc. It was found that without sufficient humidity (90–100%) the adult mites would soon die. In dry sand all *T. deliensis* were dead in 24 hr. In one cell containing normal soil in which specimens were found, all mites appeared to be dead after 24 hr. Moisture was then added and the mites were still alive 3 days later. Absence from the laboratory for several days terminated the experiments.

Five adult *T. deliensis* were placed in a glass specimen tube (1 by ¼ in.) containing a small strip of damp blotting paper and the tube corked. Twenty-five days later four of the mites were still alive and very active. No ova or larvae were observed in the tube.

In the above details the life cycle of *T. deliensis* appears to agree with that worked out by Miyajima & Okumura (1917) for *T. akamushi*. Nine days after the larvae are fully engorged the larval skin bursts and the nymph emerges. Nineteen days after the adults were placed in tubes larvae appeared.

Trombicula deliensis Walch, 1923

The adult venter (Fig. 1) shows the body to be of a figure **8** shape having a definite constriction at the anterior third. The genital pore lies in the median line posterior to coxa iv, with the anal pore still farther back. The body is thickly clothed with bipectinate setae (Fig. 3 *a*, *b*). In life the mite is pinkish white and very active.

The dorsum of the adult (Fig. 2) shows the crista to be close to the gnathosoma. The crista is shown in greater detail in Fig. 4*a*. The sensory seta is provided with a number of fine hairs (Fig. 4*b*).

Leg i longer than the remainder, legs ii and iii subequal, leg iv slightly longer than ii and iii. Tarsus i (Fig. 5) differs from the others in being ovoid, 191 μ long and 115 μ at its maximum width.

Tarsus ii, iii and iv (Fig. 6 *a*, *b* and *c*) are respectively 110 μ long, 42 μ wide; 116 μ long, 42 μ wide; 163 μ long, 42 μ wide. All legs are clothed with pectinate setae and terminated by two claws.

The mandibles (Fig. 7) are chelate and only slightly curved. The palp (Fig. 8) is five-jointed with a stout main claw and three subsidiary claws, and is clothed with several pectinate setae. The genital pore (Fig. 9) has two pairs of lips clothed with pectinate setae and possesses three pairs of disks.

Measurements. Total length (including capitulum) 1200 μ. Length of body (excluding capitulum) 900 μ. Width (anterior to constriction) 600 μ. Width (posterior to constriction) 675 μ. Length of leg i 675 μ, leg ii 450 μ, leg iii 450 μ, leg iv 630 μ. Length of crista 144 μ. Width between sensory setal bases 68 μ. Length of sensory seta 153 μ. Length of dorsal setae 74 μ. Length of chelicera 102 μ.

Table 1

	Larvae
35 bandicoots (*Bandicota malabarica* Shaw)	2148
141 rats (*Rattus r. kandianus* Kelaart)	1162
28 musk shrews (*Suncus c. giganteus* Geoffroy)	432
6 gerbilles (*Tatera i. ceylonica* Wroughton)	83

No. of hosts examined	No. without larvae
14 ♂ 11 ♀ bandicoots	3 ♀
90 ♂ 53 ♀ rats	53 ♂ 42 ♀
17 ♂ 10 ♀ musk shrews	7 ♂ 2 ♀
3 ♂ 3 ♀ gerbilles	1 ♀

Number of nymphs reared from engorged larvae

No. of days	No. of larvae	No. of nymphs	Host
9	12	3	Rat
20	18	3	Shrew
15	12	2	Rat
13	80	3	Bandicoot
13	40	3	Rat
9	29	3	Bandicoot
11	35	1	Rat
9	100	73	Bandicoot
9	20	11	Rat
11	150	16	Bandicoot
10	100	40	Bandicoot

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LARVAL AND NYMPHAL MITES (ACARINA; TROMBICULIDAE) FROM CEYLON AND THE MALDIVE ISLANDS

By CHARLES D. RADFORD, *Squadron Leader, Royal Air Force*

(With 29 Figures in the Text)

INTRODUCTION

During five months spent in Colombo (April–August 1944) a large number of small mammals and birds were examined for larval Trombiculinae.

From November 1944 to February 1945 similar investigations were carried out on the Maldive Islands. The number of species obtained included three species of the genus *Gahrliepia* Oudemans, 1912 (not included in this paper), *Trombicula deliensis* Walch, 1923, *Trombicula acuscutellaris* Walch, 1923, *Neoschöngastia indica* Hirst, 1915, *Walchia turmalis* Gater, 1932 (a second species of *Walchia* not included in this paper), a new species of *Schöngastia* herein named '*maldiviensis*' n.sp., and *Womersleyia minuta* n.g., n.sp.

Breeding experiments have been conducted and the results obtained with *Trombicula deliensis* Walch have been published already (Radford, 1945). Herein are described the larval, nymphal and adult stages of *Neoschöngastia indica* Hirst, 1915, the larval and nymphal stages of *Trombicula deliensis* Walch, 1923, the larval and nymphal stages of *Trombicula acuscutellaris* Walch, 1923, the larval and nymphal stages of *Schöngastia maldiviensis* n.sp., and the larva of *Womersleyia minuta* n.g., n.sp.

Walch (1923*b*, p. 621) figures the crista of *Trombicula muris* which is a synonym of *Neoschöngastia indica* Hirst, 1915. In Walch's figure the sensory setae are long, slender and not feathered (bipectinate).

In my breeding experiments I have successfully reared 194 nymphs from engorged larvae that were definitely *Neoschöngastia indica*, and they all possess the feathered sensory setae herein figured, as also do the adults.

In the breeding experiments the nymphs remain alive for 4–5 days and then become inert. Although they were kept for a number of weeks they did not appear to change to adults. Adults of *Trombicula deliensis* have been found in the soil, but so far the adults of the other species have not been recovered though large quantities of soil have been examined. Search was therefore made at the tops of coconut palms (*Cocos nucifera* L.) and, in the spaces between the leaves and trunk of the tree, adult *Neoschöngastia indica* Hirst were found amongst the dead flowers and leaf debris. The adults have not been described previously.

Trombiculae—standard data. In descriptions of the Trombiculinae the following letters are used to denote the standard data upon which specific characters are based:

AW = width between anterior lateral setae.
PW = width between posterior lateral setae.
SB = width between bases of sensory setae.
ASB = distance from anterior border of scutum to base of sensory seta.
PSB = distance from posterior border of scutum to base of sensory seta.
A-P = distance from anterior to posterior lateral setae.
AM = length of anterior median/medial seta/setae.
AL = length of anterior lateral setae.
PL = length of posterior lateral setae.
Sens. = length of sensory setae.
DS = number of dorsal setae (excluding dorsal scutum).
SD = scutal depth (i.e. ASB + PSB).

Trombicula deliensis Walch, 1923

The dorsum of the larva (Fig. 1) shows the shape of the dorsal scutum, also the number and arrangement of the dorsal setae. The dorsal scutum is pitted and furnished with the customary number of setae, the sensory setae are provided with side hairs on the distal half, and, flanking the scutum at the level of the posterior lateral setae, are the paired eye plates.

The venter of the larva (Fig. 2) shows the arrangement and number of ventral setae and the coxae, each provided with a single seta. The ur-stigmatic pore is shown on the venter of coxa i. The palp and chelicera are shown in Figs. 3 and 4.

Measurements of larva (standard data). AW 60μ; PW 70μ; SB 30μ; ASB 23μ; PSB 15μ; A-P 29μ; AM 27μ; AL 20μ; PL 27μ; Sens. 56μ; DS 28; SD 38μ.

The dorsal setae (not including the dorsal scutum) are arranged in rows: 2, 8, 6, 6, 4, 2.

Hosts. Ceylon house rat (*Rattus rattus kandianus* Kelaart), bandicoot (*Bandicota malabarica* Shaw), musk-shrew (*Suncus caeruleus giganteus* Geoffroy),

Fig. 1. larv
Fig. 2.
Fig. 3.
Fig. 4.

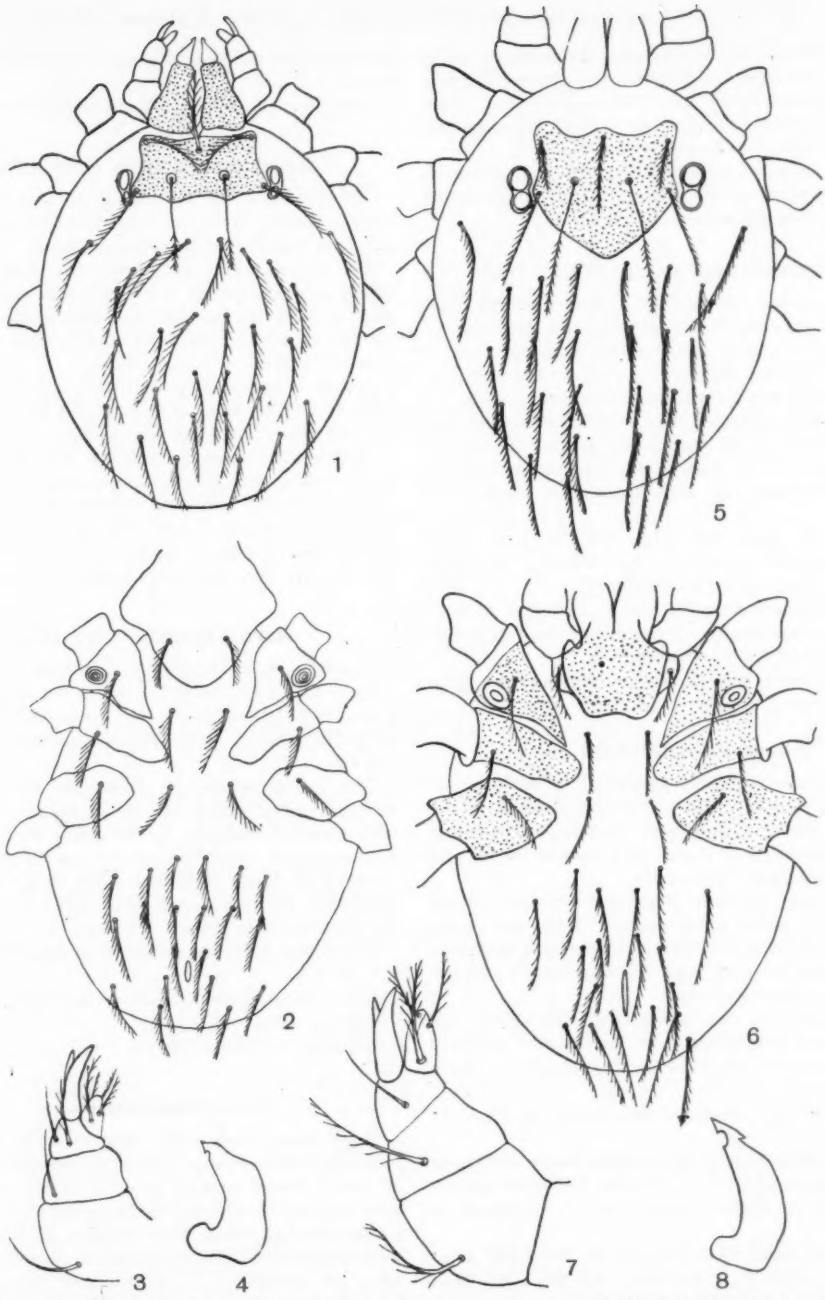

Fig. 1. *Trombicula deliensis* Walch, 1923. Dorsum of larva.
Fig. 2. *T. deliensis* Walch, 1923. Venter of larva.
Fig. 3. *T. deliensis* Walch, 1923. Palp of larva.
Fig. 4. *T. deliensis* Walch, 1923. Chelicera of larva.
Fig. 5. *T. acuscutellaris* Walch, 1923. Dorsum of larva.
Fig. 6. *T. acuscutellaris* Walch, 1923. Venter of larva.
Fig. 7. *T. acuscutellaris* Walch, 1923. Palp of larva.
Fig. 8. *T. acuscutellaris* Walch, 1923. Chelicera of larva.

gerbille (*Tatera indica ceylonica* Wroughton), palm squirrel (*Funambulus palmarum favonicus* Thomas & Wroughton), house crow (*Corvus splendens*), crow pheasant (*Centropus sinensis parroti* Stresemann), and koel (*Eudynamis scolopaceus* L.).

On the Maldive Islands. Brown rat (*Rattus norvegicus norvegicus* Berkenhout) and musk-shrew (*Suncus caeruleus giganteus* Geoffroy).

Trombicula acuscutellaris Walch, 1923

The dorsum of larva (Fig. 5) shows the arrangement and number of dorsal setae and the shape of the dorsal scutum; the surface of the scutum is pitted. Flanking the scutum, close to the posterior lateral setae, the paired eye plates can be seen.

The larval venter (Fig. 6) shows the number and arrangement of the ventral setae and the pitted integument of the base of the capitulum and the coxae. The palp and chelicera are shown in Figs. 7 and 8.

Standard data. AW 74μ; PW 78μ; SB 32μ; ASB 34μ; PSB 43μ; A-P 77μ; AM 48μ; AL 31μ; PL 54μ; Sens. 85μ; DS 26; SD 77μ.

Dorsal setae arranged in rows: 2, 6, 6, 6, 4, 2.

This species can be distinguished readily by its large size and vivid red colour.

Host. Brown rat (*Rattus norvegicus norvegicus* Berkenhout).

Schöngastia maldiviensis n.sp.

This species is closely similar to *Neoschöngastia schuffneri* Walch, 1923, but differs from it in the size and shape of the dorsal scutum, the number and arrangement of dorsal and ventral setae, and in the chaetotaxy of the palp.

The dorsum of larva (Fig. 9) shows the scutum to possess a pitted integument with the eye plates flanking the scutum. The anterior and posterior lateral setae are very long and bipectinate and the sensory setae are clavate.

The venter of the larva (Fig. 10) shows the number and arrangement of the ventral setae of which there are 26 whereas in '*schuffneri*' there are 30.

The palp and chelicera are shown in Figs. 11 and 12.

In '*schuffneri*' the palp possesses seven feathered and two plain setae. The chelicera of the new species is only 34μ in length, whilst that of '*schuffneri*' is 50μ long.

Standard data. AW 51μ; PW 68μ; SB 17μ; ASB 25μ; PSB 25μ; A-P 30μ; AM 36μ; AL 68μ; PL 60μ; Sens. 38μ; DS 30; SD 50μ.

Dorsal setae arranged in rows: 2, 8, 6, 6, 4, 4, 2. In '*schuffneri*' they are in rows: 2, 10, 10, 8, 8, 6.

When alive the larva is brick red in colour.

Hosts. Lizard (*Calotes versicolor*) and occasionally on brown rat (*Rattus norvegicus norvegicus* Berkenhout).

Locality. Gan, Maradu, Fedu Islands, Addu Atoll.

Neoschöngastia indica Hirst, 1915

The dorsum of the larva (Fig. 13) shows that the clavate sensory setae are furnished with small hairs and that the five scutal setae are short.

The venter (Fig. 14) shows the characteristic shape of the base of the capitulum and slender coxa ii, with the number and arrangement of the ventral setae. The palp and chelicera are shown in Figs. 15 and 16.

Standard data. AW 34μ; PW 53μ; SB 17μ; ASB 17μ; PSB 19μ; A-P 30μ; AM 17μ; AL 17μ; PL 21μ; Sens. 25μ; DS 32; SD 26μ.

Dorsal setae arranged in rows: 2, 8, 6, 6, 4, 4, 2. In life this species is of a dirty white colour.

Hosts. Ceylon house rat (*Rattus rattus kandianus* Kelaart), Colombo and Embelipitiya, Ceylon. Brown rat (*Rattus norvegicus norvegicus* Berkenhout), Addu Attol, Maldive Islands.

Walchia turmalis Gater, 1932

The dorsum of larva (Fig. 17) shows the dorsal scutum to possess only four setae, an anterior and a posterior lateral pair, with no anterior median seta. The sensory setae are missing but should be clavate(?).

The venter (Fig. 18) shows the number and arrangement of setae. All the coxae are furnished with a single seta each. In the genus *Walchia* some species possess more than one seta on the third coxa.

Standard data. AW 34μ; PW 51μ; SB 25μ; ASB 25μ; PSB 34μ; A-P 37μ; AM nil; AL 21μ; PL 25μ; Sens. ?; DS 48; SD 59μ.

The dorsal setae are arranged in rows: 2, 2, 6, 6, 8, 8, 6, 6, 4.

Host. Musk-shrew (*Suncus caeruleus giganteus* Geoffroy).

Locality. Colombo, Ceylon.

Genus **Womersleyia** n.g.

Small mites, only 170μ long by 119μ wide: in nature a reddish orange colour: possessing two pairs of eyes. Dorsal scutum roughly shield shape, its apex directed forwards; bearing six setae in addition to the sensory setae. The anterior medial pair of setae placed close to apex of scutum; anterior lateral setae at shoulders of scutum and the posterior lateral setae in the normal position. These six scutal setae are mounted upon papillae, furnished with a plain shaft and are bipectinate at the distal half. Sensory setae are slender with the distal half bipectinate. Dorsal setae all mounted upon papillae.

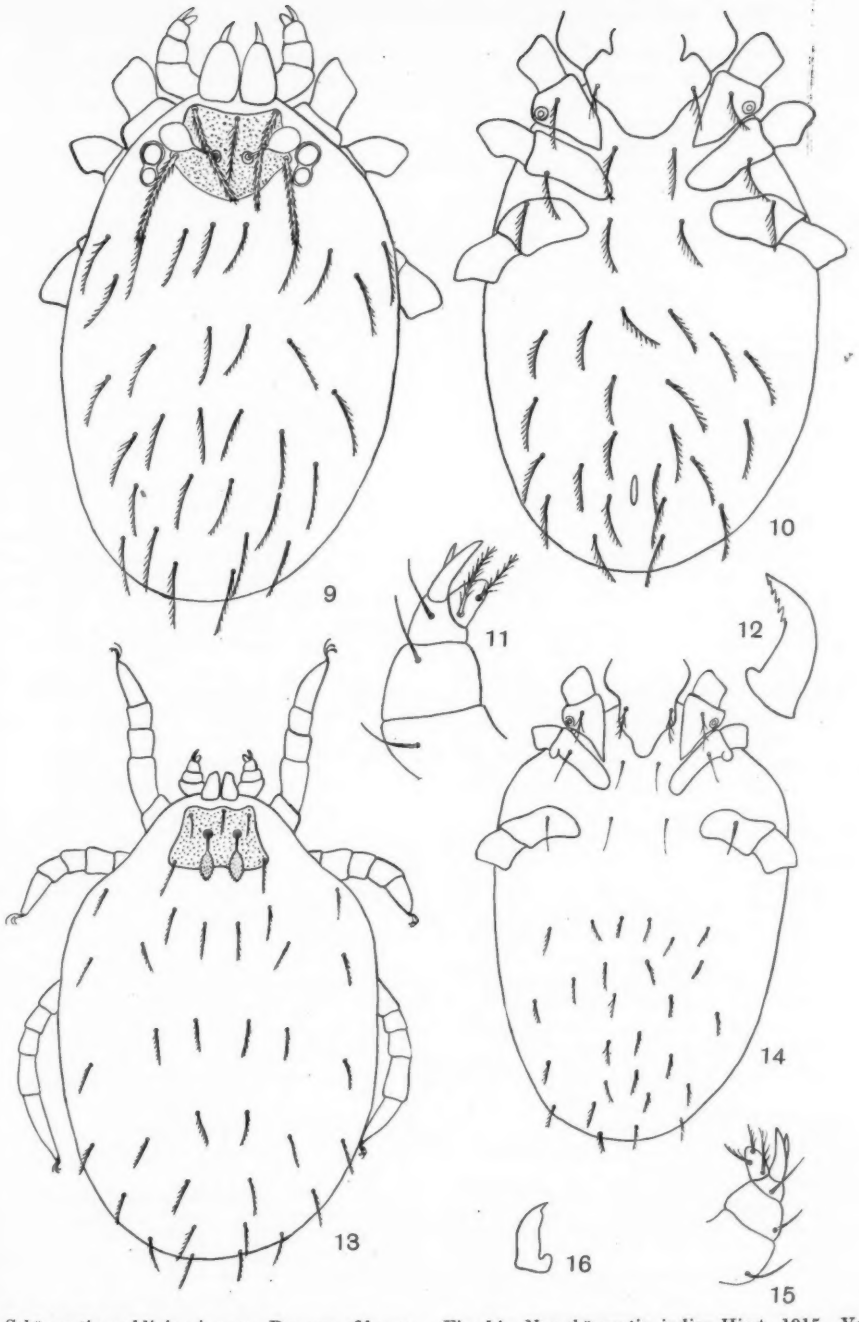

Fig. 9. *Schöngastia maldiviensis* n.sp. Dorsum of larva.
Fig. 10. *Schöngastia maldiviensis* n.sp. Venter of larva.
Fig. 11. *Schöngastia maldiviensis* n.sp. Palp of larva.
Fig. 12. *Schöngastia maldiviensis* n.sp. Chelicera of larva.
Fig. 13. *Neoschöngastia indica* Hirst, 1915. Dorsum of larva.
Fig. 14. *Neoschöngastia indica* Hirst, 1915. Venter of larva.
Fig. 15. *Neoschöngastia indica* Hirst, 1915. Palp of larva.
Fig. 16. *Neoschöngastia indica* Hirst, 1915. Chelicera of larva.

Palps furnished with two claws, and on the movable process of the palp (tarsus) there is a pair of stout setae similar in shape to the scutal setae.

Genotypes found in the mud of a pandanus swamp but subsequently found to be parasitic upon three families of grasshoppers (Acrididae, Tetrigidae

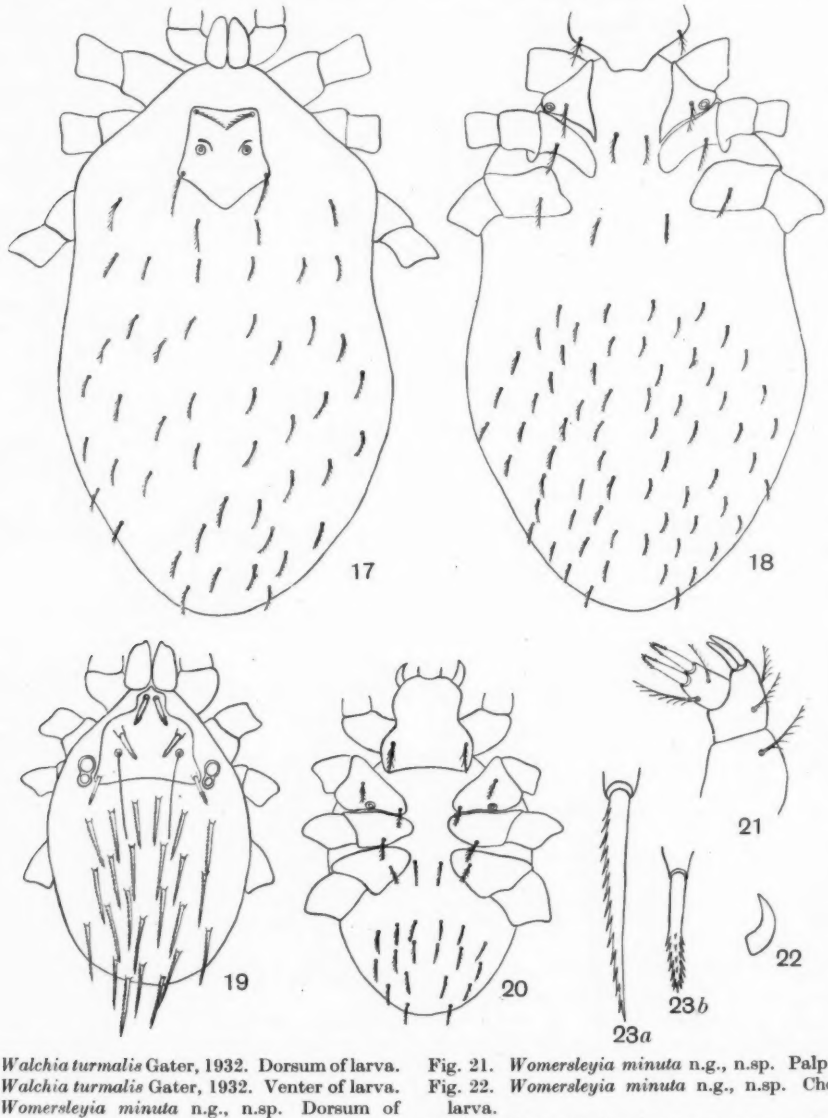

Fig. 17. *Walchia turmalis* Gater, 1932. Dorsum of larva.
Fig. 18. *Walchia turmalis* Gater, 1932. Venter of larva.
Fig. 19. *Womersleyia minuta* n.g., n.sp. Dorsum of larva.
Fig. 20. *Womersleyia minuta* n.g., n.sp. Venter of larva.
Fig. 21. *Womersleyia minuta* n.g., n.sp. Palp of larva.
Fig. 22. *Womersleyia minuta* n.g., n.sp. Chelicera of larva.
Fig. 23. *Womersleyia minuta* n.g., n.sp. *a*, dorsal seta; *b*, scutal seta.

Coxa i with two setae, coxa ii and iii each with one seta, as in the genus *Leeuwenhoekia* Oudemans, 1911.

The genus is placed in the subfamily Leeuwenhoekinae Womersley, 1944.

and Tettigoniidae). At present only the larvae are known.

The genus is named in honour of Herbert Womersley, Entomologist, South Australian Museum, Ade

laide, who has done much to further our knowledge of the Trombidiidae.

Genotype. Womersleyia minuta n.g., n.sp.

Womersleyia minuta n.g., n.sp.

The dorsum of the larva (Fig. 19) shows the characteristic shape of the dorsal scutum, and the position of the scutal setae which are all mounted upon papillae, the anterior lateral pair being the most conspicuous. Sensory setae long, slender, with distal half bipectinate. Eye plates bearing a pair of eyes are placed at the posterior lateral corners. Dorsal setae pectinate and mounted upon papillate bases.

The larval venter (Fig. 20) shows coxa i to possess two setae and coxa ii and iii to be provided with one seta each, as in the genus *Leeuwenhoekia* Oudemans, 1911.

The palp (Fig. 21) is furnished with two claws and the tarsus is terminated by two stout setae, similar to those on the dorsal scutum. The chelicera (Fig. 22) is plain and lacks dentition. Dorsal and scutal setae are shown in Fig. 23 *a*, *b*.

Standard data. AW 34μ; PW 55μ; SB 34μ; ASB 40μ; PSB 17μ; A-P 25μ; AM 17μ; AL 17μ; PL 20μ; Sens. 51μ; DS 24; SD 60μ.

Dorsal setae arranged in rows: 6, 6, 6, 3, 3.

Hosts. Parasitic (under wings) of grasshoppers (Acrididae, Tetrigidae and Tettigoniidae). Original specimens found in mud of pandanus swamp, Island of Gan, Addu Attol, 20 December 1944.

Trombicula deliensis Walch, 1923

Nymph. In general appearance the nymph is similar to the adult only smaller. In life the nymph is pale pinkish white in colour and possesses the figure **8** shaped body common to these mites.

The crista (Fig. 24*a*) shows its characteristic shape and the sensory setae are long, slender and bipectinate at the distal half. The palp (Fig. 24*b*) is furnished with one stout claw and two accessory claws with a number of pectinate and plain setae. The chelicera (Fig. 24*c*) is provided with a number of teeth and the dorsal seta (Fig. 24*d*) shows the club-shaped distal end.

Length of crista 102μ; width between sensory setae 51μ; length of sensory setae 142μ.

Schöngastia maldiviensis n.sp.

Nymph. This species differs more in the nymphal stage from *Neoschöngastia schuffneri* Walch, 1923 than it does in the larval stage. In Walch's paper (1923*b*, fig. 21, p. 601) the sensory setae of the nymph are shown to be long, slender and to possess only very short side hairs. In the present species the sensory setae are long, slender and simple, without any lateral hairs. Walch (1923*b*, fig. 23, p. 614) depicts the palp and it will be seen that in his species there are many more setae than in the present species.

Nymph. In life the nymph is reddish in colour and similar in shape to that of *T. deliensis*.

The crista (Fig. 25*a*) shows the triangular chitinized shield upon which the long, slender, simple sensory setae are borne. This triangular form of crista is found in the nymphal and adult stages of a number of species of the Trombiculinae, and Womersley has illustrated a number of them in a paper now in the press.

The palp (Fig. 25*b*) shows the single large claw and two accessory claws with the chaetotaxy of the last three joints of the palp. The chelicera and dorsal seta are shown in Fig. 25 *c*, *d*.

Length of crista 93μ; width between sensory setae 30μ; length of sensory setae 93μ.

Neoschöngastia indica Hirst, 1915

Nymph. In size and shape similar to that of *T. deliensis* but whitish in colour and differing from the adult *N. indica* in size only.

Again we see the triangular crista (Fig. 26*a*) but the sensory setae are not plain. They are long, medially expanded and bipectinate for two-thirds of their length.

The palp (Fig. 26*b*) shows the large terminal claw and the two accessory claws. The chelicera, sensory seta and dorsal seta are shown in Fig. 26 *c*, *d*, *e*.

Length of crista 93μ; width between sensory setae 32μ; length of sensory setae 68μ.

Trombicula acuscutellaris Walch, 1923

Nymph. This species is readily distinguished by its bright red colour and size, which is twice that of the other species herein mentioned.

The crista (Fig. 27*a*) shows the triangular shape, and the sensory setae are long, slender and simple (unfeathered). The palp (Fig. 27*b*) shows the large terminal claw with its two accessory claws. The chelicera and dorsal seta are shown in Fig. 27 *c*, *d*.

Length of crista 162μ; width between sensory setae 38μ; length of sensory setae 136μ.

Neoschöngastia indica Hirst, 1915

Adult. The adults of this species have not, as yet, been bred from nymphs and the specimens now described were found at the tops of coconut palms (*Cocos nucifera* L.) living in the debris of leaves and dead flowers between the trunk of the tree and its leaves. No other species of adult Trombidiidae were found in their company.

They possess three pairs of discs on the genital pore as in the adult of *T. deliensis*, whereas the nymph possesses only two pairs. One specimen when prepared was seen to have an ovum *in situ*.

In life they are whitish and have the typical figure **8** shape (Fig. 29).

The crista (Fig. 28*a*) bears a very close resemblance to that of the nymph differing in size only. The palp, chelicera and dorsal seta are shown in Fig. 28 *b*, *c*, *d*.

Of the 200 or more identified larvae at present known to science only twelve are known as nymphs or adults (i.e. as a result of breeding in the laboratory).

Hirst (1915) has reared *Trombicula autumnalis* Shaw, 1790 as far as the nymph, while Ewing (1929) has been equally successful with *T. irritans* Riley, 1873.

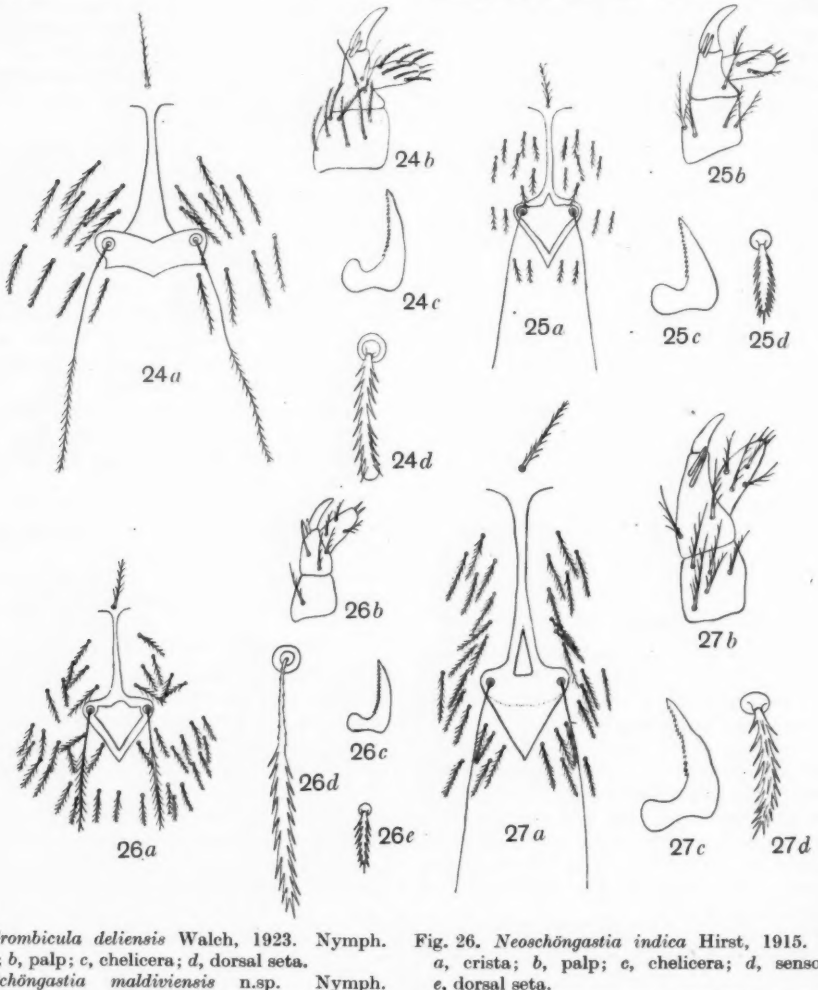

Fig. 24. *Trombicula deliensis* Walch, 1923. Nymph. *a*, crista; *b*, palp; *c*, chelicera; *d*, dorsal seta.

Fig. 25. *Schöngastia maldiviensis* n.sp. Nymph. *a*, crista; *b*, palp; *c*, chelicera; *d*, dorsal seta.

Fig. 26. *Neoschöngastia indica* Hirst, 1915. Nymph. *a*, crista; *b*, palp; *c*, chelicera; *d*, sensory seta; *e*, dorsal seta.

Fig. 27. *Trombicula acuscutellaris* Walch, 1923. Nymph. *a*, crista; *b*, palp; *c*, chelicera; *d*, dorsal seta.

Length of crista 108 μ; width between sensory setae 43 μ; length of sensory setae 93 μ.

Breeding technique

The difficulty of breeding the Trombiculinae will perhaps be appreciated when one considers how little is known about the life histories of these mites.

Nagayo, Miyagawa, Mitamura & Imamura (1916) have reared the nymphs of *T. akamushi* Brumpt, 1910. Nagayo, Miyagawa, Mitamura, Tamiya & Tenjin (1921) claimed to have reared nymphs of *T. palpalis*, *pallida*, *intermedia* and *scutellaris*. As, however, they were unable to point to any specific differences between these nymphs and

T. akamushi, no reliance can be placed upon these four records.

Kawamura & Yamaguchi (1921) described and figured the nymph of *Neoschöngastia gallinarum* Hatori, 1920, in which the sensory setae are clavate.

Walch (1923*b*) described the nymphs of *Trombicula deliensis*, *T. pseudo-akamushi* Hatori, *Neoschöngastia indica* Hirst (= *Trombicula muris* Walch) and *Schöngastia oudemansi* Walch (= *Trombicula oudemansi*).

He states (p. 601): 'For example, I was always successful in rearing *T. pseudo-akamushi* and *T. oudemansi*. I tried to rear *T. schuffneri* eight times and succeeded only once. I tried to rear *T. deliensis* eighteen times and succeeded four times. I made only two attempts to rear *T. muris* and succeeded in reaching the nymphal stage once.' And (p. 711): 'Of four Delian species the nymphae could not be reared owing to a lack of a sufficient number of larvae, namely the *Trombicula acuscutellaris*, the *T. densipiliata*, the new species *T. keukenschrijveri* of which we only possess one specimen, and the *T. rara* of which we only possess four.'

To this number must be added *Trombicula minor* Berlese, 1904 and *T. samboni* Womersley, 1939. Gunther (1939), working in New Guinea, reared nymphs of *T. hirsti* var. *bulolensis* Gunther, which has since been identified with *T. minor* Berlese.

Melvin, working in Panama, succeeded in rearing the nymphs of two species, *Eutrombicula hominis* Ewing, 1933 and one, as yet unidentified, species.

Kohls, working in New Guinea, reared the nymphs of *Trombicula fletcheri* Womersley, 1943, *T. scincoides* Womersley, 1944, *Guntherana bipygalis* Gunther, 1939, *Neoschöngastia impar* Gunther, 1939, *N. womersleyi* Gunther, 1940, *Neoschöngastia* n.sp. and *Leeuwenhoekia novaguinea* Womersley, 1944.

The nymphs of *Trombicula deliensis* Walch were reared in Colombo, using the following technique.

The ears of rodents which possessed larvae were cut off and placed in 3 by 1 in. glass specimen tubes containing a mixture of sand and dead leaves, damped with a few drops of rain water. After 24 hr. the majority of the larvae would have left the ears and burrowed into the soil. The ears were removed and the tube again corked. A daily examination showed that activity ceased by the second day and 9 days after preparation of the cell the first nymphs appeared. These nymphs remained active for 2 or 3 days and were not again seen though the cells were examined.

Adults, which proved to be *T. deliensis* Walch, 1923, were found in the soil around the burrows of rats and bandicoots. To rear the larvae from these adults a different method was deemed advisable. The convexity of the 3 by 1 in. glass tubes made examination difficult so staining cells (cavity blocks, glass, 37 mm.sq.) were used. The sealing of the glass lids to these cells with vaseline produced a growth of mould inside the cell in which the adult mites became trapped. This necessitated the adoption of

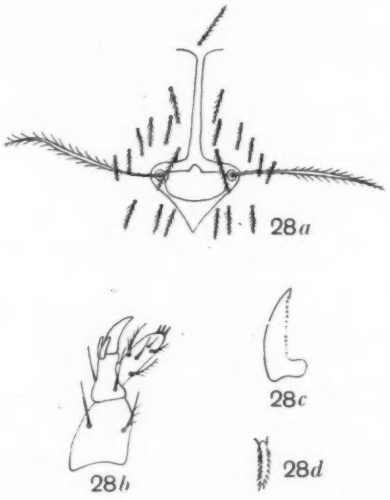

Fig. 28. *Neoschöngastia indica* Hirst, 1915. Adult. *a*, crista; *b*, palp; *c*, chelicera; *d*, dorsal seta.

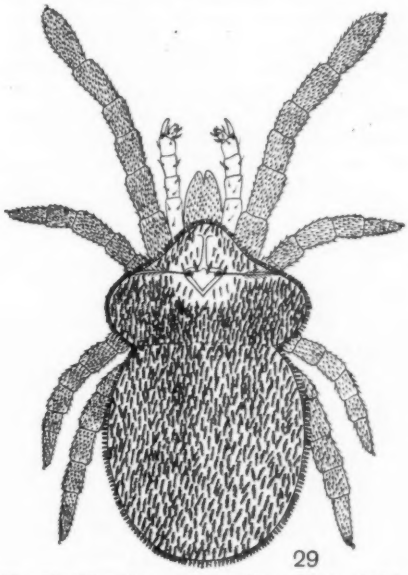

Fig. 29. *Neoschöngastia indica* Hirst, 1915. Adult. Dorsum.

a new breeding cell cover, which was made from scrap aluminium, 1½ in. square and $\frac{3}{16}$ in. thick. A 1 in. hole was cut in the centre and this was covered with a piece of bolting silk glued on. The same mixture of sand and leaf mould was used and 19 days after the adults were placed in the cell the first larvae appeared. Upon examination these proved to be *T. deliensis*. No ova were seen although the cells were examined daily. In some of the adults, however, one egg was seen, never more.

The same type of cell proved unsuccessful for the experiments at Gan, Addu Attol, in the case of *T. deliensis*. Glass-topped tin pill boxes were then tried but without success.

Using the 3 by 1 in. tubes for the larvae of *Schöngastia maldiviensis* n.sp. the author succeeded in rearing the nymphs. One attempt to rear the nymphs of *Trombicula acuscutellaris* Walch also proved a success and several attempts to rear the nymphs of *Neoschöngastia indica* Hirst were successful. All further attempts, however, were unfruitful.

The technique used by Kohls in New Guinea was tried and appears to be satisfactory. Using test-tubes 6 by ¾ in. in which 3 in. or more of the tube was occupied by the mixture (dead leaves and sand), water was added and the rats' ears were placed in the tubes. The day following, the ears were removed and the tubes plugged with cotton-wool.

These tubes were then stood upright in a tin or box some 5 or 6 in. high to prevent the light from getting below the level of the soil. In nature the larvae burrow down into the soil away from the light, and the failure to rear nymphs whilst using the 3 by 1 in. tubes and again in using the glass-topped tin pill boxes, is thought to be due, to some extent, to the admission of too much light into the cells.

The results obtained by this method were very gratifying and nymphs of *Neoschöngastia indica* Hirst were produced after 6 and 8 days. Nymphs of *Trombicula acuscutellaris* Walch were seen walking about in the tubes on the ninth day.

Attempts to rear *Neoschöngastia indica* Hirst to the nymphal stage succeeded on nine and failed on three occasions. *Trombicula acuscutellaris* Walch has been successfully reared to the nymphal stage on two occasions and ten attempts failed. Thirteen efforts to rear *T. deliensis* Walch all failed.

The following table gives the number of animals trapped together with the number of larvae:

Host	No. examined	No. with larvae ♂	No. with larvae ♀	No. without larvae ♂	No. without larvae ♀	No. of larvae	No. per host
Colombo. 30 April 1944 to 3 October 1944							
Bandicoot	30	18	7	1	4	2985	119·4
Rats	164	35	12	69	48	1244	26·4
Shrews	28	11	8	7	2	432	22·7
Gerbilles	6	3	2	—	1	83	16·6
Addu Attol. 6 November 1944 to 28 February 1945							
Rats	278	106	119	17	36	9033	40·3
Shrews	250	79	40	97	34	2939	25·1

This paper has been made possible by the untiring efforts of my assistant, Pte J. Hake, R.A.M.C., who, under very trying conditions, has shown no diminution in his zeal and ingenuity in devising traps and making the trapping so successful.

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NAIROBI SHEEP DISEASE: THE SURVIVAL OF THE VIRUS IN THE TICK *RHIPICEPHALUS APPENDICULATUS*

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(With 6 Figures in the Text)

INTRODUCTION

Montgomery (1917) showed that Nairobi sheep disease, a tick-borne gastro-enteritis of sheep and goats in East Africa, was caused by a filtrable virus transmitted most commonly by the brown tick, *Rhipicephalus appendiculatus*. The transmission of the disease by this tick was confirmed by Daubney & Hudson (1931, 1934), who demonstrated also that ticks infected in any instar usually transmitted the disease when fed in the succeeding stage on susceptible sheep; and that the virus ingested by a female fed on an infected sheep passed through the eggs to the larvae of the next generation.

In laboratory experiments, transmissions have hitherto been obtained with ticks fed shortly after their emergence from the moult or from the eggs. The fate of the virus in ticks kept unfed for long periods had not been determined. Montgomery, however, described an experiment carried out in a paddock where sheep, infested with *R. appendiculatus*, died of Nairobi sheep disease. After the last death, the paddock was left unoccupied for 9 months. At the end of this period, susceptible sheep were introduced, and they contracted the disease presumably from ticks that had dropped off, 9 months previously, from sheep which had died of Nairobi sheep disease.

Dealing with preventive measures against the importation of the disease on to farms where *R. appendiculatus* exists, Montgomery recommended the quarantine of doubtful sheep for at least 21 days. He stated also that if deaths occurred, the quarantine should be regarded as infective, and though the sheep could be removed without fear, the area should be kept free of sheep and goats for 18 months. No experimental data or field evidence are given to support this last recommendation.

The following account of additional experiments conducted at Kabete shows that the virus of Nairobi sheep disease will survive in a virulent state in ticks unfed up to 871 days.

THE VIRUS IN ADULT TICKS INFECTED AS NYMPHAE

Nymphae of *R. appendiculatus* from a series fed on rabbits in the larval stage were infected on sheep 5464 in which Nairobi sheep disease was provoked by the inoculation of virulent blood. The nymphae had attached to the sheep prior to the inoculation. They became engorged during the reaction, and dropped replete within 24 hr. after the temperature had subsided. The reaction lasted 6 days. The replete nymphae moulted to adults in 20–23 days.

When the adults were 136 days old, five males and five females were put to feed on sheep 5471 which, like all other sheep used for the transmissions in these experiments, was a grade Merino. The male ticks attached readily, but the females were slow in settling down. Consequently the first engorged female did not drop off the sheep until the twelfth day after infestation. The temperature of the sheep

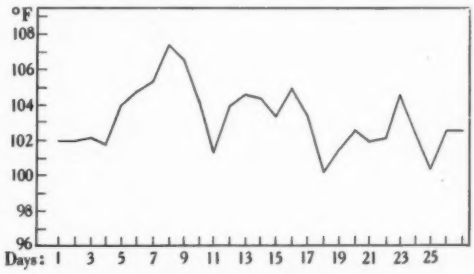

Fig. 1. Temperature reaction, sheep 5741.

remained normal for 4 days. On the fifth, it rose from about 102·0 to 104·0°F. It continued to rise on the following 2 days, and reached 107·4°F. on the eighth day after infestation. The reaction was then tested by subinoculations of the blood, and proved to be due to Nairobi sheep disease. The virus had survived in the ticks for 156 days. Incidentally, the subsequent temperature (Fig. 1) of sheep 5741 may be of interest. A remission on the eleventh day was followed by what appeared to be a second, milder reaction, and a longer remission followed by another, more irregular, elevation of temperature. No subinoculations were made during these phases, and the temperature returned to normal on the twenty-eighth day.

The remainder of the adults derived from nymphae that had engorged on reacting sheep 5464 were kept unfed for 212 days, and then fed on sheep 5717. No reaction resulted from this infestation, but the sheep was later shown to be immune to

Nairobi sheep disease. Another series of nymphae was infected on sheep 5217. These ticks were reared from larvae that had fed on a sheep (no. 4763) reacting to Nairobi sheep disease. The resulting infective nymphae seemed unlikely to survive the desired period of starvation, and many died. Approximately 200 of the healthy survivors were, therefore, fed on sheep 5217 to which they, as nymphae, transmitted the disease. They continued to feed during the reaction that they themselves had provoked. They engorged on infective blood; and the following transmission by the resultant adults confirms the findings of Daubney & Hudson (1931).

Ten adult ticks, starved for 395 days, were put to feed on sheep 5462 which gave a typical reaction to Nairobi sheep disease. Another batch of ten adults from the same lot, starved for 528 days in this case, was fed on sheep 5474 to which the ticks transmitted the disease, the course of which calls for no comment. Still another batch of the same number of adults, 618 days old, was put to feed on sheep 5601. The ticks attached within a few hours. Although they fed normally, the incubation period of the disease induced was slightly longer than in the infections provoked by adults used previously from the same source. A final batch of thirty adults, 720 days old, failed to infect sheep 5844 which, however, subsequently proved to be immune.

An older series of adults, infected as nymphae on a diseased sheep, contained a small number of survivors just over 2 years after moulting. It was divided into two lots. One, comprising ten males and ten females, was fed on sheep 5740 when the ticks were 755 days old; and the other, of the same number of adults, was fed on sheep 5848 when the ticks were 851 days old. The disease transmitted to sheep 5740 was of the characteristic acute form of Nairobi sheep disease and the sheep died. Sheep 5848 gave a typical temperature reaction (Fig. 2) followed by recovery. Allowing for the moulting period, the virus had retained its virulence in these ticks for 775 and 871 days respectively.

THE VIRUS IN MALE TICKS

It is not unusual, though perhaps not very common, for a male *R. appendiculatus* to detach itself from one host and to find its way on to another. It seems to occur more frequently on woolly sheep than on hairy sheep or cattle. The male feeds before mating, moves from its original site of attachment to search for a female. After mating, the female engorges and drops to the ground whereas the male seeks another female or re-attaches to feed. The writer has observed that a male failing to find another female wanders away from the small, hairy ears, and the perineum and strays over the thick coat of wool. When the sheep are closely paddocked or packed together, the passage of stray ticks from animal to animal is not difficult. The role of male ticks in the transmission of Nairobi sheep disease seemed, therefore, worth investigating.

Clean males and females were put to feed on sheep 6942 at the time of its reaction to Nairobi sheep disease induced by blood inoculation. All the ticks were attached at the end of 24 hr. Within a few days, the males had completed the first meal and had moved on to the females. When the fertilized replete females had dropped, the males re-attached and appeared to be feeding for 4 days after the temperature of the sheep had subsided. The males were removed and transferred to a rearing tube in which they were kept until they showed signs of dying off. Out of twenty-two males, six were alive on the thirteenth day in the tube. These, with six clean females, were fed on sheep 6909 which reacted to Nairobi sheep disease and died 16 days after infestation.

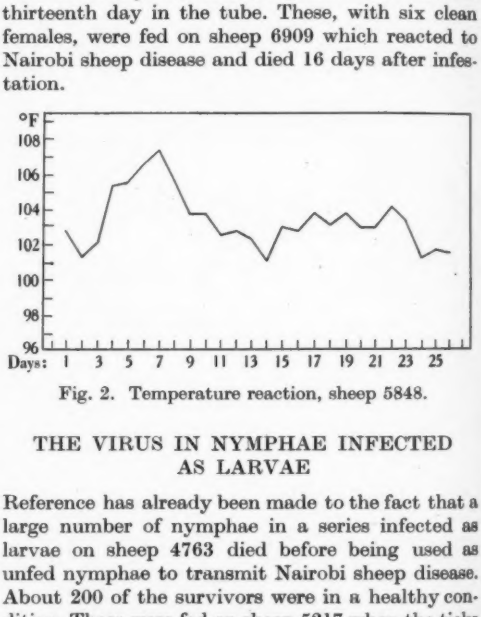

Fig. 2. Temperature reaction, sheep 5848.

THE VIRUS IN NYMPHAE INFECTED AS LARVAE

Reference has already been made to the fact that a large number of nymphae in a series infected as larvae on sheep 4763 died before being used as unfed nymphae to transmit Nairobi sheep disease. About 200 of the survivors were in a healthy condition. These were fed on sheep 5217 when the ticks were 128 days old. They transmitted the disease with an acute thermal reaction culminating in the death of the animal. The engorged larvae moulted to nymphae in 10 days so that the virus transmitted by the nymphae had survived for 138 days in the ticks.

Another series of nymphae infected as larvae lived for a slightly longer period. Sheep 7212 was infested with a batch of 200 nymphae, 114 days old; and sheep 7216, which had previously failed to react to larval ticks, was infested with twenty nymphae 288 days old. Both lots of nymphae transmitted Nairobi sheep disease. Sheep 7212 died after an acute attack, whereas sheep 7216 recovered from a

fever lasting 7 days. A second successful transmission was obtained with nymphae, 288 days old, fed on sheep 7240 which, like sheep 7216, had failed to react to an earlier infestation by larvae reared from an infective female. The nymphal ticks used in these three instances were active, and eager to feed; but others from the same series were unable to withstand longer periods of starvation so satisfactorily. While in the tubes, the survivors were to all appearances lively and strong; but when transferred to sheep, the majority failed to attach and died. About fifty nymphae were put on sheep 7251 after the ticks had been starved for 327 days. Forty-three were found dead on the following day; seven had attached, and of these only three succeeded in feeding to repletion. These three nymphae dropped on the seventh day. On the following day, the temperature of sheep 7251 (Fig. 3) rose sharply to 105·8°F., but dropped to normal (99·8° F.) on the tenth day. Blood drawn from this sheep on the one day of high temperature was inoculated into two other sheep and produced a febrile reaction conforming with the classical type of Nairobi sheep disease. At 348 days after moulting, only eighteen nymphae of this series were active. When they were put to feed on sheep 7223, sixteen failed to attach. Two had apparently pierced the skin and were alive at the end of the first day. On the third day, one of these was dead, but the other was obviously feeding; and it dropped replete on the morning of the fourth day. The temperature of the sheep rose from 101·4°F. on the fourth to 107·4°F. on the fifth day. It remained at about this high level until the eighth day after which it returned to normal. The sheep recovered from the disease tested, and confirmed as Nairobi sheep disease, by subinoculations.

These experiments show that the virus of Nairobi sheep disease can survive for at least 359 days in unfed nymphae kept alive in the laboratory.

THE VIRUS IN LARVAE REARED FROM INFECTED REPLETE FEMALES

Sheep 5927, 5936 and 5942 were infested with clean adults of *R. appendiculatus*. When all the ticks had attached, the sheep were infected with Nairobi sheep disease by the inoculation of virulent blood. The ticks fed well; and the sheep reacted.

The first replete females dropped while the temperature of the sheep was elevated; and the last females dropped during a slight secondary reaction on the eleventh and twelfth days after infestation. Oviposition by the females, incubated at 25°C. (77°F.), was good; and the larvae hatched out from 32 to 38 days after the females had dropped off the sheep.

Sheep 6009 was infested with many hundreds of these larvae 79 days after they had hatched; and over 600 engorged larvae were recovered. The sheep showed a temperature (104·8°F.) on the fourth day, and died of acute Nairobi sheep disease 3 days later. Sheep 5485 was infested with a larger number of larvae 144 days old. About 2000 engorged larvae were collected from the ears of this sheep which gave a typical reaction to the disease, and recovered. The next large batch of larvae, starved for 186 days before infesting sheep 5578, did not feed so well. Only a few had attached by the end of the first day, and the vast majority had died. Even those that had attached did not appear to be feeding. No

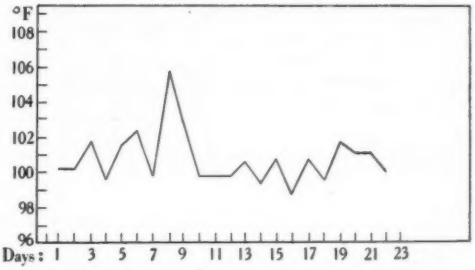

Fig. 3. Temperature reaction, sheep 7251.

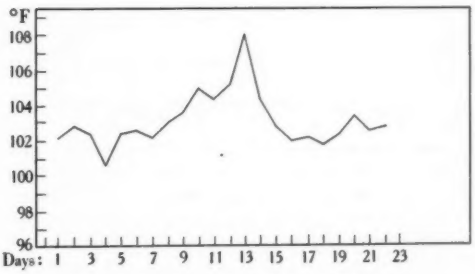

Fig. 4. Temperature reaction, sheep 5578.

replete ticks were recovered. The ears of the sheep were carefully examined every morning; and it is unlikely that any replete ticks escaped from the ear-bags. Unfortunately, the larvae that had died while remaining attached were not examined in order to determine whether or not they had actually succeeded in obtaining a small meal of blood. They did not appear to have pierced the superficial blood vessels. Nevertheless, the disease was transmitted to the sheep. The incubation period (Fig. 4) was longer than usual. The temperature began to rise (from 102·0 to 103·0°F.) on the eighth day. It continued to climb steadily and, with a slight remission on the eleventh when the reaction was tested by subinoculations of the blood, the highest temperature (107·0°F.) was registered on the thirteenth day. The temperature then fell rapidly; and the

sheep recovered from what was proved to be Nairobi sheep disease.

Two more lots each containing numerous larvae from two infected females were used to infest sheep 7181 and 7182 after the ticks had been starved for 245 days. Although a large proportion died without attempting to feed, many larvae had attached by the end of the first day. Many of the latter were dead on the next day; but eleven replete larvae were recovered from sheep 7181 on the sixth and seventh days, and four replete larvae from sheep 7182 from the sixth to the eighth day after infestation. No definite febrile reaction resulted in either of the sheep. On the fourteenth day, however, the temperature of both sheep gave a short abrupt jump from 100·2 to 103·2°F. in sheep 7181 and from 100·6 to 103·2°F. in sheep 7182. Relatively slight elevations of this kind (Figs. 5, 6) are not infrequent

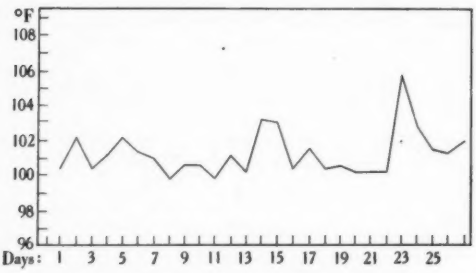

Fig. 5. Temperature reaction, sheep 7181.

in experimental animals kept in stalls; and considered in this light, the increased temperature of these two sheep is not significant; but since the incubation period of the disease in sheep 5578 seemed to have been extended as a result of the age (186 days) of the larvae, and the maximum temperature occurred on the thirteenth day, it was suspected that in sheep 7181 and 7182 the incubation period had again been prolonged as a result possibly of an attenuation of the virus in larvae 245 days old. It was considered unlikely that heartwater had been transmitted by larvae of *R. appendiculatus*. The history of the ticks reared through several generations in the laboratory, and the history of the animals, contained no record of heartwater. Microscopic examination of the blood of the two sheep revealed no *Anaplasma* or *Babesia*. Nairobi sheep disease was naturally suspected; and on the fourteenth day, blood drawn from sheep 7181 was inoculated subcutaneously into sheep 7228 which gave a similar thermal rise on the thirteenth day. Further subinoculations in series resulted in the disappearance of this suspicious mild reaction. Subsequent inoculations of known infective blood into sheep 7181 and 7182 produced febrile reactions which in sheep 7181 lasted for 1 day, and in sheep 7182 comprised two peaks of 104·8 and 103·8°F. separated by a remission on two consecutive days. These reactions also were tested. They proved the disease to be Nairobi sheep disease; and both sheep were later shown to be immune.

Whole broods of larvae from infected females were put, 294 days after hatching, on both ears of sheep 7216 and 7240 before these animals were used for transmission of Nairobi sheep disease by nymphae (pp. 56–7). Many of the larvae were seen probing the skin, but all were found dead in the ear-bags 18 hr. later. The sheep did not react to the disease to which they were afterwards shown to be susceptible.

Although larvae of *R. appendiculatus* can be kept alive in the laboratory up to 300 days (Lewis, 1939), very large numbers that survived for over 294 days

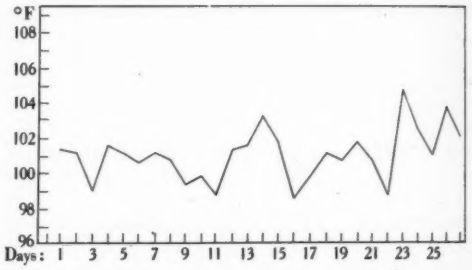

Fig. 6. Temperature reaction, sheep 7182.

in these experiments were unable to find, or to pierce, the superficial blood vessels. The ability of ticks in such a state to transmit the virus by injecting it into the blood stream is obviously affected, although the virus itself may be viable. Further experiments to test the virulence of the virus in ticks weakened by starvation are now being carried out by the inoculation of emulsified ticks. The fate of the virus in dead ticks is also being studied.

CONCLUSIONS

It is clear from the experiments described that the virus of Nairobi sheep disease will survive in *R. appendiculatus* for periods up to 871 days. This survival contrasts strikingly with that of *Theileria parva* which, as Lewis & Fotheringham (1941) have shown, disappears from infected hungry ticks of the same species in about 300–365 days. The period for which an area of land infested with infected *R. appendiculatus* may remain dangerous for sheep susceptible to Nairobi sheep disease is, therefore, longer than the 18 months' freedom from sheep and goats recommended by Montgomery. Indeed, it appears that the virus of Nairobi sheep disease, once it is

introduced into an area where *R. appendiculatus* exists, will persist so long as unfed infected ticks remain on the land.

The writer records with gratitude the advice and guidance given by Mr R. Daubney, C.M.G., O.B.E., Director of Veterinary Services, Kenya Colony; and the valuable assistance of Mr A. J. Wiley, Laboratory Assistant; and of Hezekiah Rator, Native Assistant at the Veterinary Research Laboratory, Kabete.

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RHIPICEPHALUS NEAVEI WARBURTON, 1912 AS A VECTOR OF EAST COAST FEVER

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(With 4 Figures in the Text)

INTRODUCTION

The incidence of east coast fever in areas where the disease is endemic is associated with the common occurrence of the usual tick vector, *Rhipicephalus appendiculatus*. Outbreaks of the disease in other areas are sporadic or seasonal, and although, in many such instances, *R. appendiculatus* has been found either in isolated localities, or very sparsely distributed over the whole of an infected area, in other cases it has not been possible to collect even a single specimen of this tick. Collections of ticks from infected farms in these sporadic areas comprise such species as *Boophilus* spp., *Rhipicephalus capensis*, *R. simus* and *R. evertsi* or *Hyalomma* spp., *Amblyomma* spp., *Rhipicephalus pulchellus* and *R. neavei*.

The ability of most of these other species to transmit *Theileria parva*, the causal organism of east coast fever, has been investigated by laboratory experiments, but that of *Rhipicephalus neavei* has not. Its incrimination as a vector under laboratory conditions, as we shall show, is of interest in that the natural distribution of the tick frequently overlaps with that of *R. appendiculatus*, and extends widely into areas where *R. appendiculatus* is apparently unable to survive. *R. neavei* may, therefore, serve to maintain infection in some sporadic areas, and may contribute to periodic outbreaks of the disease beyond the borders of the scheduled east coast fever endemic areas. But this tick is also commonly found on cattle in large areas from which no cases of east coast fever have been reported and where the disease is believed not to exist. This observation, coupled with Zumpt's (1942) conclusion that *R. neavei* is an atypical form of *R. appendiculatus* but one and the same species, adds to the interest and value of continued studies of east coast fever and its potential vectors.

Large numbers of ticks were collected off grass in the tsetse-infected areas of Kibwezi and Makueni in the Machakos district of Kenya Colony. They included a few *R. appendiculatus*, many *R. simus* and related species, and numerous adults of a *Rhipicephalus* which, according to Warburton's description, were unmistakably *R. neavei*. These ticks were brought to the Veterinary Research Laboratory, Kabete, where the *R. neavei* were carefully separated and put to feed on cattle and rabbits. Many engorged females were obtained from these animals; and large supplies of larvae, nymphae and adults were reared. The adults, thus reared, were again examined and the morphological characters left no doubt of the identity of these ticks with *R. neavei* as described and illustrated by Warburton.

TRANSMISSION BY NYMPHAE INFECTED AS LARVAE

Larvae of *R. neavei* were infected on bovine 04023 reacting to east coast fever transmitted by nymphae of *R. appendiculatus*. The larvae were put on the ears of the bovine on the fifth day of the febrile reaction, and they fed over a period of 11 days. Engorged larvae began to drop on the sixth day after infestation when piroplasms were common in smears made from the blood of the bovine. Seven hundred and thirty fully fed larvae were recovered before the animal died of east coast fever. These were incubated at 77·0–78·8° F. (25–26° C.), and they moulted to nymphae in 11–12 days.

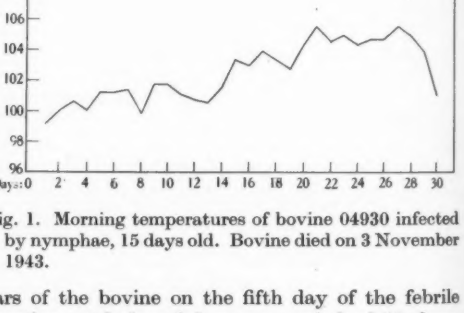

Fig. 1. Morning temperatures of bovine 04930 infected by nymphae, 15 days old. Bovine died on 3 November 1943.

One batch of 100 nymphae from this series of ticks infected as larvae were put to feed, 15 days after moulting, on a susceptible grade bull calf (04930). The ticks did not feed well and only ten fed to repletion. The others died without having attached. The bovine showed (Fig. 1) a definite rise of temperature (101·6–103·4° F.) on the fifteenth day after infestation, and the fever continued for 15 days.

Koch's bodies were rare in smears taken from the ear gland on the first day of the temperature reaction, but they became more common up to the sixth day, after which smears were made from tissue of the prescapular gland where the Koch's bodies were present in very much smaller numbers. Bovine 04930 died on the thirtieth day after infestation. Post-mortem examination showed slight hyperaemia of the fourth stomach, considerable yellowing of the medullary region of the kidneys, and enlarged oedematous lymphatic glands. The spleen was normal, but smears prepared from the tissue contained a fairly large number of Koch's bodies.

A second batch of 100 nymphae derived from the larvae infected on bovine 04023 was fed on a grade calf (04967) on the same day as bovine 04930 was infested. In this instance, also, the number (11) of replete ticks recovered was disappointing. But bovine 04967 reacted after an incubation period of 14 days. Koch's bodies and piroplasms became more frequent in gland and in blood smears, respectively, as the disease progressed. The animal died of east coast fever on the thirty-third day after infestation. The results of the post-mortem were identical with those in the case of bovine 04930. It is worth noting that neither of these calves (04930 and 04967) showed typical lesions of east coast fever when examined after death. No ulcers were seen in the fourth stomach, nor were there infarcts in the kidneys. The spleen in the former was not enlarged and only slightly enlarged in the latter. Smears prepared at death from glands, spleen and blood contained numerous gamonts, agamonts and piroplasms of *Theileria parva*.

In a third experimental transmission by infected *Rhipicephalus neavei* from the same batch of nymphae, an adult grade steer (04381) was infested with nymphae that had been starved for 64 days after moulting. Only twenty nymphae fed to repletion. Many died on the day following infestation, and many dropped off later when partly fed. On the fourteenth day, the temperature of bovine 04381 showed a tendency to rise (99·6–102·2° F.). Following a remission on the next day, it rose to 103·2° F. and continued at a high level (see Fig. 2), with a maximum of 105·2° F. until the twenty-fourth day after infestation. The temperature returned to normal on the twenty-fifth day. Koch's bodies had been fairly common in gland smears taken on the fourth to the eighth day of the reaction; but thereafter they became more rare and, ultimately, disappeared. Piroplasms in the blood were always rare or very rare. Bovine 04381 recovered and, when later tested with a virulent strain of *Theileria parva* in *Rhipicephalus appendiculatus*, proved immune to east coast fever.

The successful transmission of east coast fever by nymphae of *R. neavei* fed, as larvae, on an animal reacting to the disease originally transmitted by *R. appendiculatus* indicates the role possibly played by *R. neavei* in areas where this species of tick overlaps, or meets, with *R. appendiculatus*. It was desirable also to determine the ability of *R. neavei* to maintain infection in the absence of *R. appendiculatus*. The experiments were, therefore, adjusted with a view to establishing infection, if possible, by serial passage of *Theileria parva* from one phase to another of *Rhipicephalus neavei* rather than from *R. appendiculatus* to *R. neavei*. Unfortunately, we encountered difficulties with the feeding of larval *R. neavei* which had not been met with earlier in the experiments. The initial feeding of larvae on the ears of cattle had been reasonably successful. The numbers fed to repletion ranged from 627 to 1232 from rather small broods reared in the laboratory. These successes were followed by the failure of

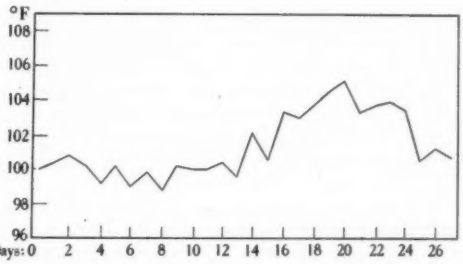

Fig. 2. Morning temperatures of bovine 04381 infected by nymphae, 64 days old. Bovine recovered.

almost all subsequent broods to feed satisfactorily. The reason for these failures has not yet been determined.

One of the few exceptions was that of clean larvae put to feed on bovine 04930 during reaction provoked by nymphae of *R. neavei*. In this case 370 replete larvae were recovered. The nymphae from these larvae were divided into two lots, and after a starvation period of 30 days, they were fed on bovines 05422 and 05360—both fully grown grade steers. The nymphae fed well. The two bovines reacted on the fifteenth and the fourteenth days respectively, and the fever, maintained at a high level, lasted 19 and 17 days. Koch's bodies and small piroplasms were seen early in the febrile reaction, and increased to considerable numbers as the disease developed. Both animals died on the thirty-fifth day after infestation. Post-mortem examination revealed typical lesions of east coast fever with ulcers in the stomach, infarcts in the kidneys, and enlarged oedematous lymphatic glands. Apart from the fact that typical post-mortem lesions were found in these animals, the disease showed no significant difference from that in the three bovines mentioned in the earlier experiments described in this article.

TRANSMISSION BY ADULTS INFECTED AS NYMPHAE

All the ticks used in the following experiments were fed, as larvae, on rabbits, and as nymphae, on cattle reacting to east coast fever transmitted by *R. neavei*.

One lot of clean nymphae was put to feed on bovine 04381 on the fourth day of the 'mild' reaction when Koch's bodies had been found in fairly large numbers in gland smears. Piroplasms in the blood were, as already stated (p. 61), always infrequent, and the animal recovered. The nymphae fed satisfactorily, and began to drop on the ninth day of fever. The replete ticks continued to drop up to 5 days after the reaction temperature of the bovine had subsided. They were put into an incubator to moult, and the adult ticks emerged in 10–17 days.

The moulted adults were starved for 32 days, and one batch of 100 were then used on a susceptible bovine 04424, a fully grown grade heifer. The ticks attached readily, and many engorged females were recovered. The temperature of the bovine remained normal until the twelfth day when a slight rise (101·4–102·6° F.) was registered. Following a remission on the next day, the temperature resumed its upward tendency on the fourteenth, and remained at about 103·4° F. until the death of the animal on the eighteenth day. Koch's bodies appeared in gland smears on the first day of the reaction or 12 days after infestation. They were rare for the first 3 days, but fairly common on the fourth. At death, on the sixth day of fever, these bodies were numerous both in the glands and in the spleen. Parasites were scarce in the blood stream even when the animal died. The post-mortem revealed no changes characteristic of east coast fever. The actual cause of death could not be determined although lesions were present suggestive of heartwater. But there is no doubt that the adult *R. neavei* transmitted east coast fever.

The experiment was repeated, and two lots of infective adults from the same source fed on two grade steers (04440 and 06185) provoked a reaction conforming with that produced by infective *R. appendiculatus*. The incubation period in both cases was 13 days. On the fourteenth day, a thermal rise (103·2 and 105·6° F.) indicated the onset of fever. In bovine 04440, the reaction continued for 16 days and culminated in death. Koch's bodies and small piroplasms occurred in abundance 6 days after the first rise of temperature, and post-mortem examination showed the usual ulcers, infarcts, tumor splenis, swollen oedematous glands, and the sequelae of this disease. In bovine 06185, the reaction lasted 11 days. The temperature was often above 105·0° F. (Fig. 3) whereas in bovine 04440 it reached this height once only. The initial rise in the former was more acute, and death was more sudden.

It is evident from these few experiments that nymphae of *R. neavei* will readily take up the parasite of east coast fever, and transmit the disease in the adult stage in very much the same way as *R. appendiculatus*. It was, however, necessary to test the survival of the parasite in *R. neavei*, and to determine whether ticks of this species kept in the laboratory remained infective as long as, or longer than, *R. appendiculatus*. A number of adults, fed as nymphae on reacting bovine 05360, were kept for 262 and 269 days. They survived starvation reasonably well.

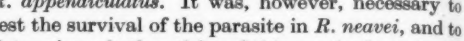

Fig. 3. Morning temperatures of bovine 06185 infected by adult ticks, 39 days old. Bovine died on 24 January 1944.

The first lot, 262 days old, contained twelve males and twenty females. These were put on a grade bullock (06002). They attached readily, and nine females fed to repletion. The temperature of the bovine began to rise on the fifteenth day, and increased progressively with frequent slight remissions, to 106·6° F. on the twenty-eighth day. It then subsided and the animal died 2 days later. Koch's bodies and piroplasms in smears taken at intervals were common and were numerous immediately before and after death. On post-mortem, the fourth stomach showed no ulcerations, but numerous petechiae were present. The kidneys contained numerous infarcts, the liver was enlarged, the spleen normal, and the lymphatic glands were large and oedematous.

The second lot of adult *R. neavei* 269 days old, comprised twenty males and thirty-five females. When put on another grade bullock (07114) they attached within 24 hr., and fed well. Twenty-nine females dropped replete. The temperature of the bovine remained normal for 18 days and on the nineteenth, a short rise was registered. No further increase was recorded on the next 2 days. After a maximum of 103·0° F. on the twenty-second, the temperature returned to normal (Fig. 4) and the animal recovered from a very mild reaction. Koch's bodies could be demonstrated only on the fourth day of 'fever', but piroplasms, which were fairly common in blood smears taken on that day, were still present on the twenty-sixth when, to all appear-

ances, the animal had recovered. A week later, however, bovine 07114 died. No ulcers or infarcts were seen at post-mortem, and there was nothing, but general anaemia, to indicate the cause of death.

ATTEMPTED TRANSMISSION BY LARVAE REARED FROM FEMALES FED ON INFECTED CATTLE

Several attempts were made to determine whether or not, in case of *R. neavei*, the parasite of east coast fever would pass from females engorged with infected blood through the eggs to the larvae of the next generation. The attempts were unsuccessful. The larvae refused to feed on the cattle although other series of larvae, used in biological studies, fed to repletion on cattle at about the same time. Further attempts at transmission with larval ticks were abandoned until the reasons for their periodic failure to feed on the ears of cattle could be ascertained.

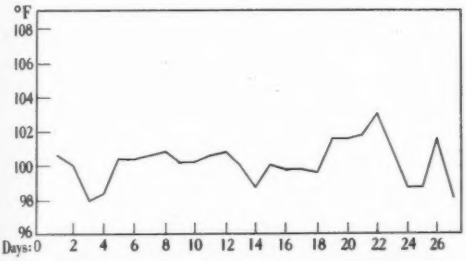

Fig. 4. Morning temperatures of bovine 07114 infected by adult ticks, 269 days old. Bovine recovered.

DISCUSSION

We have shown that the tick recognized in Kenya Colony as *R. neavei* described by Warburton (1912) is capable under laboratory conditions of transmitting east coast fever, that transmission can be effected by nymphae and adults infected as larvae and nymphae respectively, and that the adults can harbour the parasite for at least 269 days.

The incubation period of the disease provoked by *R. neavei*, in these preliminary experiments, is rather longer than is usually observed in experimental transmissions of east coast fever by *R. appendiculatus*. Apart from this difference, which may disappear when more animals are used, the disease transmitted by both species of ticks is similar. *R. neavei*, reared and fed at the laboratory may, therefore, be considered as efficient a vector of the disease as *R. appendiculatus*. The larvae of *R. neavei* did not feed on cattle as consistently and successfully as larvae of *R. appendiculatus* usually do. Sometimes they fed well, at other times they failed completely. It seems, therefore, that though cattle may, periodically, become infested with these immature ticks, they are not always suitable hosts. If this is the case in nature also, *R. neavei* is probably not as important a natural vector as *R. appendiculatus*. Nevertheless, it is reasonable to assume that under favourable conditions in the field, *R. neavei* can maintain and spread east coast fever either in the presence or in the absence of the usual vector.

Examples of the concurrence of the two species are the Karati forest near Naivasha in the so-called 'clean' area of Kenya; the Taveta district south-east of Mt Kilimanjaro; the north-west section of the Narok district of the Masai Reserve from whence east coast fever is reported to be spreading eastwards; the southern portion of the Ukamba Reserve between the Thwaki and the Kibwezi rivers, and between the Athi and the Tiva rivers; and the Kapenguria area of the Turkana Province in the north of Kenya. Several areas in Uganda and in Tanganyika are known to be similarly infested, and may be included in what may be termed the marginal zones or areas of seasonal infections. Among the areas in which *R. neavei* is common and widely distributed, and where *R. appendiculatus* appears to be unable to thrive, are the Loita Plains in the Masai Reserve where the game of the open plains, savannah and forest are favourable hosts. The cattle and sheep, in this seasonal grazing area, are also infested with *R. neavei*. The Wa-Galla stock, and the game, in the dry scrub country between the Tiva river and the Somalia (Italian Somaliland) border invariably provide numerous specimens of this tick. It occurs extensively on both sides of the Tana river and in the semi-desert areas of Garissa, Garba Tulla, Bura and Kipsing in the Northern Frontier Province, and as far north as Lake Rudolf and the Oripoi Valley in the north-west corner of Kenya Colony. It extends into the Karamoja district of Uganda, and is frequently found in parts of Tanganyika.

Practically all the areas, in the last group, that are in Kenya are situated outside the scheduled enzootic east coast fever country; and no outbreaks of the disease have, so far, been reported among the cattle of the nomadic tribes that inhabit these relatively low and warm regions. Does the disease exist in parts of these areas, or does it exist in a mild form? The susceptibility of the cattle brought from these areas into infected districts tends to show that this is not so, and, therefore, that *R. neavei* in these vast regions is not a carrier of the parasite of east coast fever. But, according to Zumpt (1942), *R. neavei* is merely an atypical form of, but the same species as, *R. appendiculatus*. If this is accepted, then the association of endemicity of east coast fever with the common occurrence of *R. appendiculatus* must be reviewed, and the whole of Kenya

must be regarded as potentially endemic unless there is some agency, other than the tick itself, that governs the development and transmission of the parasite.

We are of the opinion that the East African *R. neavei* is morphologically distinct from, but biologically closely allied to, *R. appendiculatus*. Evidence in support of this opinion, as well as the results of our study of factors that influence the development and survival of *Theileria parva* in the tick-host will be published in the near future.

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OBSERVATIONS ON A MONOCYSTID GREGARINE, *APOLOCYSTIS ELONGATA* N.SP., IN THE SEMINAL VESICLES OF *EISENIA FOETIDA* (SAV.)

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(With 45 Figures in the Text)

CONTENTS

	PAGE
I. Introduction	65
II. Material and methods	65
III. Description of the parasite	65
(1) Trophozoites	65
(2) Encysted stages	67
IV. Notes on the chromosome cycle	68
V. Systematic position of the gregarine	73
VI. Summary	73
References	74

I. INTRODUCTION

Brandling worms, *Eisenia foetida* (Sav.), collected from a rubbish heap in an old garden in Clifton, Bristol, from November 1942 to January 1943, were found to have their seminal vesicles infected with a very large acephaline gregarine, the trophozoites of which showed unusually energetic writhing movements, were covered with close-set ectoplasmic 'hairs', and had no anterior differentiation or epimerite.

All but four of the sixty-nine worms examined were thus parasitized, and generally the infection was very heavy. No other species of gregarine was observed. This state of affairs may be due to the isolated habitat of the brandlings in a garden at least a century old and, for the last thirty years certainly, subjected to little horticultural change. The occurrence of what may be described as a 'pure culture' of a large and abundant gregarine, easily recognizable at all stages of its development, suggested that it might afford suitable material for study of the chromosome cycle.

The following gregarines have been recorded from brandlings (*Eisenia* (*Helodrilus*) *foetida* (Sav.)) by other workers: *Apolocystis lumbrici-olidi* (Schmidt), *A. gigantea* Troisi, *Monocystis agilis* Stein, *M. arcuata* Boldt, *M. densa* Berlin, *M. ventrosa* Berlin, *M. suecica* Berlin, *M. herculea* Bosanquet, *Nematocystis plurikaryosomata* Bhatia & Chatterjee, *Rhynchocystis porrecta* (Schmidt), *R. piriformis* Berlin, and *Zygocystis suecica* Berlin. Among these species, *Apolocystis gigantea* most closely resembled the gregarine in the Bristol worms. But on p. 73 will be given the reasons that seem to justify our addition of a new species, *Apolocystis elongata*, to the already formidable list.

II. MATERIAL AND METHODS

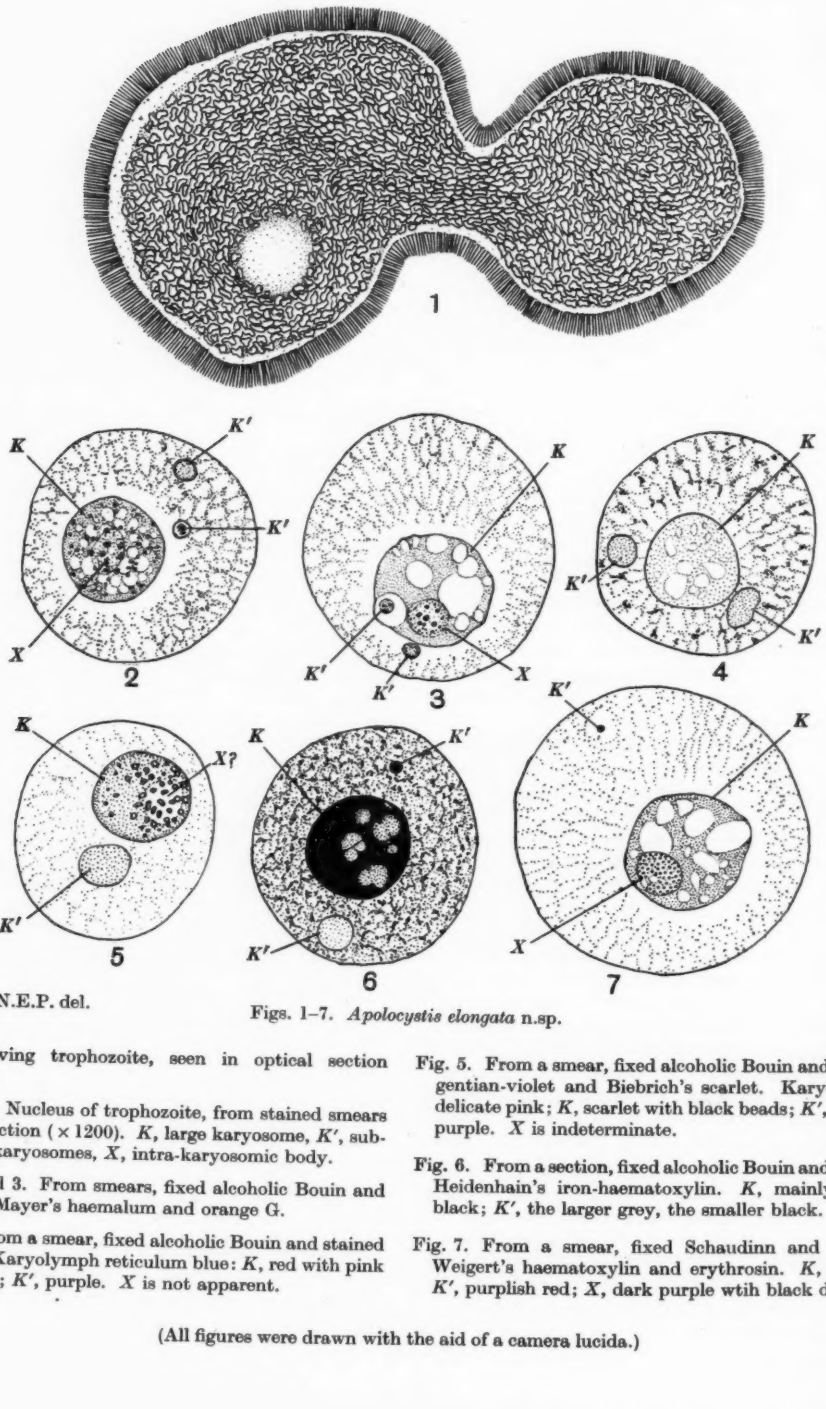
Prolonged observations were made of living material at all stages of development. Measurements of trophozoites were taken only when the organism was fully extended and free from pressure: gametocysts were measured and camera lucida drawings were made of these before the cover-glass was lowered.

Smears fixed with Schaudinn's fluid or Duboscq's alcoholic modification of Bouin's fluid were stained as follows: Delafield's haematoxylin and eosin; Mayer's haemalum and orange G; Weigert's haematoxylin with erythrosin; Mann's stain; gentian violet with Biebrich's scarlet. These preparations gave the best pictures of the trophozoite and of the nuclear structure at that stage.

Most of the cytological work, however, was based on serial sections of seminal vesicle selected for the density of infection: the gametocysts were so large that they could be seen with the naked eye. Of the fixatives used —alcoholic Bouin, Schaudinn, Carnoy, and Carnoy with chloroform—the first gave the best results. Delafield's haematoxylin was found useful, but Heidenhain's iron-haematoxylin was the stain chiefly employed: a short method (20 min. in the mordant and an hour in the stain) was fairly satisfactory, but greater clarity of detail was obtained by differentiation after a longer method (3–4 hr. in the mordant and 12 hr. in the stain). Eosin, erythrosin, Biebrich's scarlet, light green, and orange G were all tried as counterstain, but they added little of value to the picture. The material was embedded in paraffin and sectioned at 6μ. Some difficulty was experienced in avoiding distortion of the sporocysts, which are more than commonly impermeable in this species: a chloroform bath was tried but gave no better results than did the more direct method.

III. DESCRIPTION OF THE PARASITE

(1) *Trophozoites* (Fig. 1). Extreme dimensions observed were 103×33 and $220 \times 95 \mu$. No young stages were seen. The parasites were remarkable for



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Figs. 1–7. *Apolocystis elongata* n.sp.

Fig. 1. Living trophozoite, seen in optical section (× 540).

Figs. 2–7. Nucleus of trophozoite, from stained smears and a section (× 1200). *K*, large karyosome, *K'*, subsidiary karyosomes, *X*, intra-karyosomic body.

Figs. 2 and 3. From smears, fixed alcoholic Bouin and stained Mayer's haemalum and orange G.

Fig. 4. From a smear, fixed alcoholic Bouin and stained Mann. Karyolymph reticulum blue: *K*, red with pink vacuoles; *K'*, purple. *X* is not apparent.

Fig. 5. From a smear, fixed alcoholic Bouin and stained gentian-violet and Biebrich's scarlet. Karyolymph, delicate pink; *K*, scarlet with black beads; *K'*, pinkish purple. *X* is indeterminate.

Fig. 6. From a section, fixed alcoholic Bouin and stained Heidenhain's iron-haematoxylin. *K*, mainly dense black; *K'*, the larger grey, the smaller black.

Fig. 7. From a smear, fixed Schaudinn and stained Weigert's haematoxylin and erythrosin. *K*, purple; *K'*, purplish red; *X*, dark purple wtih black dots.

(All figures were drawn with the aid of a camera lucida.)

their energetic peristaltic movements and densely granular endoplasm. When most lively, they might be described as sausage-shaped, but with constantly changing outline. Only when activity was slowing down did they become oval or rounded: then they generally showed a small nipple-shaped projection at one pole.

The body was covered uniformly with stiff, close-set ectoplasmic processes, about 3μ in length. The pellicle was very thin. The clear ectoplasmic layer below this was about 4μ thick, on the average, but might extend to 7μ where the body was constricted. The endoplasm was crammed with tiny curved flakes, about 1μ in width, presumably of paraglycogen: their outline was elliptical, with marginal irregularities. The great abundance of this food reserve often obscured the nucleus completely, and its internal structure could never be studied in the living gregarine. The diameter of the nucleus in a trophozoite 200μ long was somewhere about 30μ.

Attempts were made to analyse the movements of the active parasite. (1) If the wave of contraction started at one end of the body, it passed along the whole length and then returned. (2) If contraction started in the middle of the body's length, waves moved in opposite directions towards both ends simultaneously: as these reached the ends, the paraglycogen granules recoiled towards the starting-point of the waves, where by now the diameter of the body was at its maximum. Neither of these methods led to progression. But (3) if the contraction started between the middle of the body and one of the ends, then, as in (2), the waves of contraction moved in opposite directions and, since the smaller portion of the body necessarily completed its outgoing phase of contraction before the larger, in the recoil phase its contents overtook and, so to speak, reinforced the directional thrust given by the other end. If this were repeated a number of times, the effect was to propel the organism slowly along.

The exceptionally vigorous movements of species of *Apolocystis* in brandlings, to which other observers also refer, may well be related to the very confined space in which the trophozoite lives. The seminal vesicles of *Eisenia foetida* are relatively small, whereas the parasite is much bulkier than are most of the species of monocystid gregarines parasitizing the larger earthworms, such as *Lumbricus*. Moreover, owing to the density of the infection and the large size of the gametocysts, which presumably accumulate during the life of the host, the available space is much encroached on; so, if the gregarine is to stir up the viscous medium on which it depends for food, it must exert itself energetically. Troisi (1933) described trophozoites of *Apolocystis gigantea* attached to the walls of the seminal vesicles: this was never observed in the species from our brandlings, even in sectioned material.

Since Hesse (1909) attached importance to the staining reactions of the trophozoite's nucleus in *A. lumbrici-olidi* (Schmidt), we applied a variety of staining techniques. The following is a translation of Hesse's observations on this point: 'The nucleus almost always contains two karyosomes. One of them, irregular and vacuolated, is generally stained violet by Mann, or occasionally reddish violet with a violet cap (calotte). The other is stained red by the same method; it is perfectly spherical and contains a central vacuole in the middle of which there is a red granule; it is much less bulky than is the other. These two karyosomes are sometimes in contact, or they may be more or less distant from one another and even may be placed at the two extremities of the same diameter of the nucleus. The spherical karyosome stains intensely with iron-haematoxylin, while the other takes up the stain very slightly. There may be only one karyosome: in such a case this is spherical, vacuolated and stains reddish violet with Mann. The very distinct linin network bears tiny chromatic granules distributed over its whole surface.'

We found that in the nucleus of our trophozoites the extra-karyosomic reticulum stained uniformly blue with Mann. There were always two, and sometimes there were three, 'karyosomes' (Fig. 4). A large, excentric karyosome was always present: its average diameter was 15μ. After Mann's stain it was bright red with a few pink vacuoles. The two much smaller bodies stained purple with Mann: the one more constantly present was homogeneous and stained intensely, the other stained much more faintly and sometimes showed traces of internal structure. It appears therefore that, so far as this test is concerned, Hesse's large karyosome behaved as do the small ones in our gregarine, while his small karyosome stained as does the large one in our species.

Fig. 5 shows the nucleus stained by gentian violet and Fig. 6 by iron-haematoxylin. Staining with Weigert's haematoxylin (Fig. 7) and with Mayer's haemalum (Figs. 2 and 3) revealed an intra-karyosomic body, *X*, in the large karyosome. Possibly this represents the most essential chromatinic material of the nucleus: but we have no conclusive evidence.*

(2) *Encysted stages.* Of *A. lumbrici-olidi* (Schmidt) Hesse (1909) says: 'Je ne connais rien du développe-

* Of the trophozoite nucleus in *Apolocystis gigantea*, Troisi (1933) observes: 'It is commonly spherical, averaging 35μ to 43μ in diameter, and in its centre is a large spherical karyosome measuring 19μ to 23μ in diameter. The karyosome is usually homogeneous but may appear vacuolated. About one-half of the specimens show, in addition to the karyosome, a small spherical body, probably lipoid in nature, within the nucleus.' Of staining reactions he says nothing.

ment de ce parasite.' Troisi (1933) briefly gives his observations on the association of living gamonts of his species *A. gigantea*: he also mentions the living isogametes and describes the sporocysts.

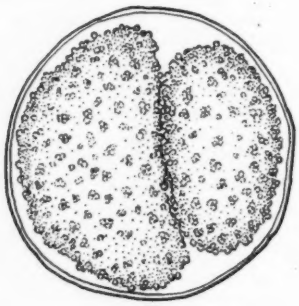
In *A. elongata* the gamonts were usually of unequal size (Fig. 8): the average dimensions of the larger were $285 \times 190\,\mu$, and of the smaller $255 \times 160\,\mu$. The ectoplasmic processes had entirely disappeared, and the surface of the gamonts was uneven. Occasionally, three gamonts lay within one gametocyst. Ripe gametocysts filled with sporocysts averaged about $460\,\mu$ in diameter: but cysts of only $255\,\mu$ diameter were seen, and one, unusually elongated, measured as much as $1244 \times 1006\,\mu$. Solitary encystment might account for the abnormally small, and triple-gamont encystment for the abnormally large specimens.

Before gamete formation each gamont became deeply lobed, and finally the outer ends of the lobes separated off as spherules with diameter varying between 40 and $80\,\mu$ (Fig. 9). Each spherule contained a dense residuum, which became covered with transparent gametes; of these there were ultimately vast numbers in each gametocyst. Very commonly, one gamont completed gamete formation before the other. The gametes all seemed to be alike, subspherical in form and about 5μ in diameter. Their liberation from the residuum was observed: their movement was slow and gliding.

Fig. 10 (drawn from a section of a gametocyst) shows the gametes stained by Heidenhain's iron-haematoxylin. The nucleus lies towards one pole, and most of the cytoplasm seems contracted around it, the remainder being tenuous and vacuolated. The nucleus always shows a karyosome and some peripheral chromatinic material in small blocks. In Fig. 11 fertilization is in progress. The vacuoles of the gametes have fused to give a single large vacuole. The pronuclei still show their karyosomes, but the extra-karyosomic material has become more diffuse.

A more advanced stage is shown in Fig. 12, where most of the zygotes are now uninucleate and are assuming the elongated shape of the sporoblast. The karyosome of the zygote is disappearing, and chromosomes are discernible. The single large vacuole in the cytoplasm tends now to disappear or to break up into smaller vacuoles.

The boat-shaped sporocysts (Fig. 13) were of very constant size, $16 \times 8\,\mu$. Nuclei could easily be seen in the living sporocyst, and their number counted. After the octonucleate stage had been reached, the cytoplasm became much more dense and the outlines of the sporozoites could not be seen. Figs. 14 and 15 show them as they appear in stained sections. There was no sporocystal residuum.



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Figs. 8, 9. *Apolocystis elongata* n.sp.

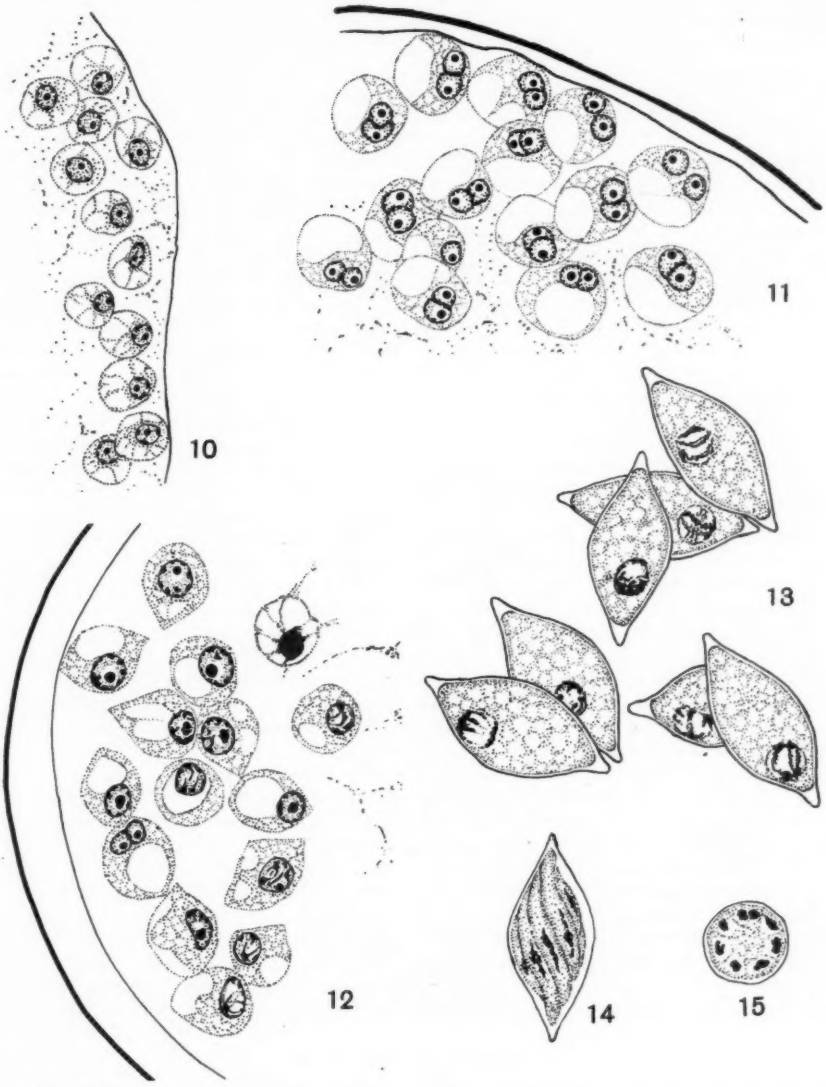
Fig. 8. Living gametocyst containing associated gamonts (× 100).

Fig. 9. Living gametocyst with contents dividing up into spherical portions each covered with gametes (× 100).

(Drawn with the aid of camera lucida.)

IV. NOTES ON THE CHROMOSOME CYCLE

The chromosomal constitution of the telosporidian sporozoa has been a matter of dispute for a good many years now. In the case of all such coccidia as have been studied intensively from this aspect, there seems no doubt that the organisms are haploid and that the reduction division is zygotic. This is said to be true also of the only schizogregarine, *Mattesia dispora*, in which the chromosome cycle has been worked out (Naville, 1930). For eugregarines, the evidence is still contradictory. Mulsow (1911),



Figs. 10–15. *Apolocystis elongata* n.sp.

Stages in spore formation. From sections of seminal vesicle fixed in alcohol Bouin, stained Heidenhain's iron-haematoxylin. Drawn with help of camera lucida.

Fig. 10. Gametes on surface of gamont (× 1150).

Fig. 11. Periphery of gametocyst with paired gametes fused (× 1150).

Fig. 12. Periphery of gametocyst with zygotes. In all but one zygote the gamete nuclei have fused, and the majority are becoming boat-shaped. A residual nucleus is shown in upper right-hand corner (× 1150).

Fig. 13. Uninucleate sporocysts (× 1875).

Fig. 14. Mature sporocyst with sporozoites formed (× 1875).

Fig. 15. Oblique section of mature sporocyst passing through the eight sporozoite nuclei (× 1875).

Trégouboff (1918), Calkins & Bowling (1926) and Naville (1927 *a*, *b*) have demonstrated that certain of these gregarines are diploid and their reduction division pregametic. Dobell & Jameson (1915), Jameson (1920), Valkanow (1935) and Weschenfelder (1938), working on other species, have taken the opposite view: according to them, eugregarines are haploid and their reduction division is zygotic.

It is possible, of course, that both conditions exist within the eugregarine group. In Naville's view, the haploid condition exhibited, for instance, by *Diplocystis schneideri* (see Jameson, 1920), is the more primitive state of affairs: the diploid condition, which Naville (1927) claims for *Urospora lagidis* and for three species of *Monocystis* in *Lumbricus*, is said to be a more recent development. The chromosome reduction in such forms takes place, according to Naville (1931), during the terminal pregametic divisions of nuclei in the gamonts: the pairing of chromosomes, which he admittedly found in the zygotes of *Urospora lagidis*, are interpreted by him as functionless vestiges of an ancestral method now discarded.

So far as can be judged from the material examined, *Apolocystis elongata* behaves as does *Diplocystis schneideri* and those other eugregarines in which the reduction division occurs at the first division of the zygote nucleus. Unfortunately, we have found no stage showing clearly the first pregametic division in the gamont. Figs. 16–25 show the state of affairs at what is probably the fourth or fifth of these divisions. The spindles, which were about 9μ long at metaphase, were provided with broad centrosome plates at the poles: there was no centriole. The asters were very conspicuous and tended to divide before the chromosomes had clearly appeared at prophase. Fig. 16 shows a nucleus in prophase, with aster about to divide: there appear here to be eight chromosomes, but study of subsequent phases shows these to be chromatids, with members of a pair abnormally far apart. Figs. 17–21 illustrate metaphase (in Fig. 20 the metaphase plate is seen in polar view). In this series the spindle was extending, and the now separated chromosome halves had become more compact. They lengthened again at anaphase (Figs. 22–25). The chromosomes were of unequal length: at metaphase they appeared as one pair of V-shaped bodies, two pairs of medium-sized rods, and one pair of very much smaller rods—these last were often mere dots and rather difficult to detect. The karyosome, very conspicuous at prophase, disappeared during metaphase, but had been reformed by the time anaphase was well advanced. As these preparations had been stained with iron haematoxylin, the relation of this karyosome to the karyosomic complex of the trophozoite could not be determined.

It should be clear from these figures that, at the fourth or fifth nuclear division in the gamont of *Apolocystis elongata*, the chromosome number is four. The material contained many examples of later pregametic divisions, but, with rapid decrease in size of the spindles, counting of chromosomes was difficult and unreliable. Naville might not unreasonably suggest, therefore, that we were failing to note a pregametic reduction division. However, our intensive study of the nuclear division in the zygote should dispose of such an objection. Figs. 26–42, gathered from an abundant material at this stage, seem to us conclusive. It is hard to believe that an unprejudiced observer could interpret what they exhibit as anything but meiosis.

The nucleus of the sporoblast lay towards one pole in a more densely granular part of the now finely vacuolated cytoplasm. When chromosomes first appeared they were rather compact, and they often had conspicuous swellings at the ends where they were in contact with the scarcely discernible nuclear membrane (Figs. 26 and 27). It was easy to count them: there were eight. They lengthened. Figs. 28–31 show progressive stages in what we interpret as leptotene. Pairing followed: Figs. 32–34 show the zygotene stage. Fig. 35 suggests pachytene, and Fig. 36 diplotene. In Figs. 37 and 38 condensation of the rearranged chromatin is taking place (late diplotene or diakinesis?). It was noticeable that the number of sporoblasts with nucleus in first-division prophase was relatively large, a fact in itself suggestive of meiosis. Fig. 39 shows pro-

Legends to Figs. 16–25

Fourth or fifth nuclear division in the gamont. From sections of seminal vesicle fixed alcohol Bouin and stained Heidenhain's iron-haematoxylin. Drawn with help of camera lucida ($\times$ 2100).

Fig. 16. Prophase. Eight elongated chromosomes (chromatids) are shown. Karyosome conspicuous.

Fig. 17. Early metaphase. Eight elongated chromosomes (chromatids) on equator of spindle. Karyosome reduced.

Figs. 18, 19. Metaphase. The eight chromosomes (chromatids) have become more compact. Spindle is elongating. Karyosome has disappeared.

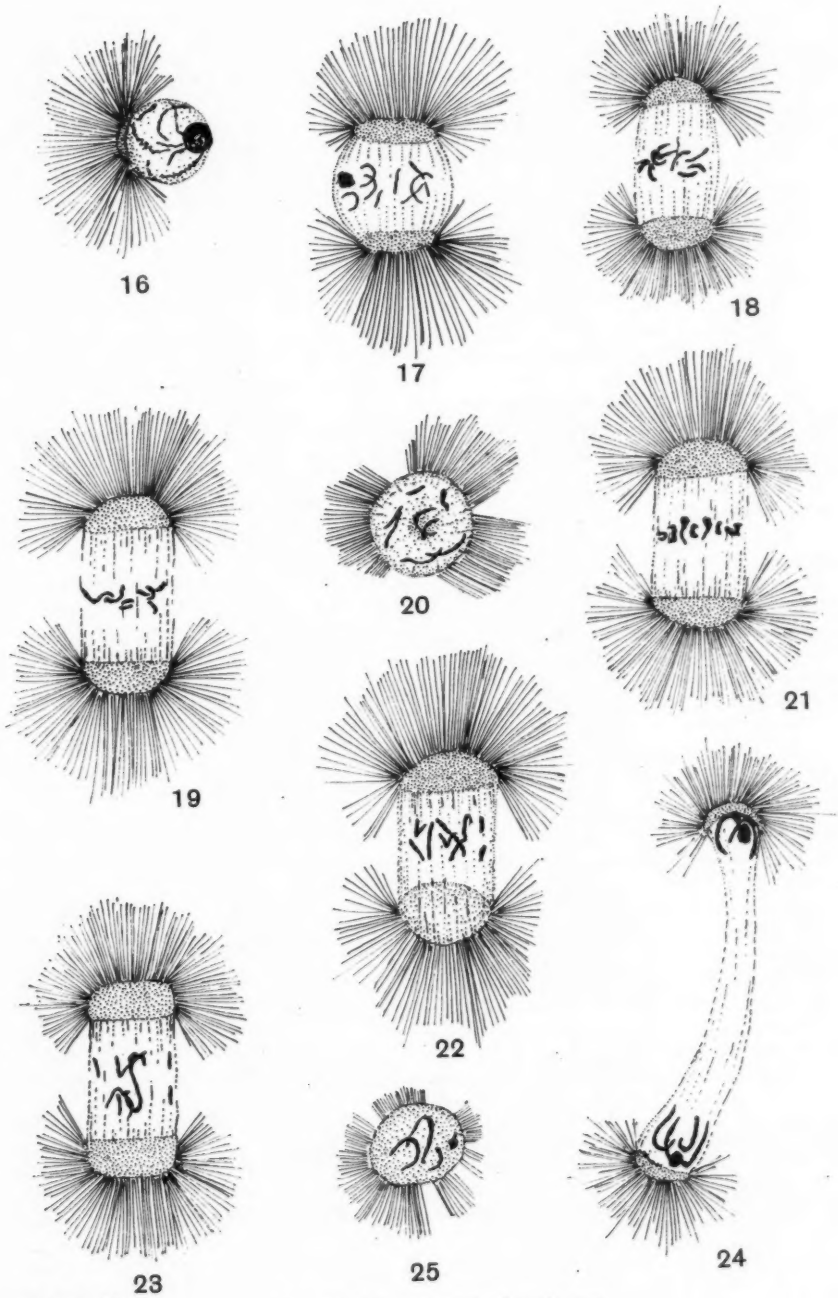
Fig. 20. Polar view of metaphase plate. The characteristic forms of the chromosomes are well seen.

Fig. 21. Late metaphase. The chromosomes (chromatids) are condensed.

Figs. 22, 23. Early anaphase. The chromosomes are again elongating, and four are on their way to each pole.

Fig. 24. Late anaphase. Four chromosomes at each pole of the elongated spindle. The karyosome has reappeared.

Fig. 25. Polar view of the four chromosomes at anaphase.



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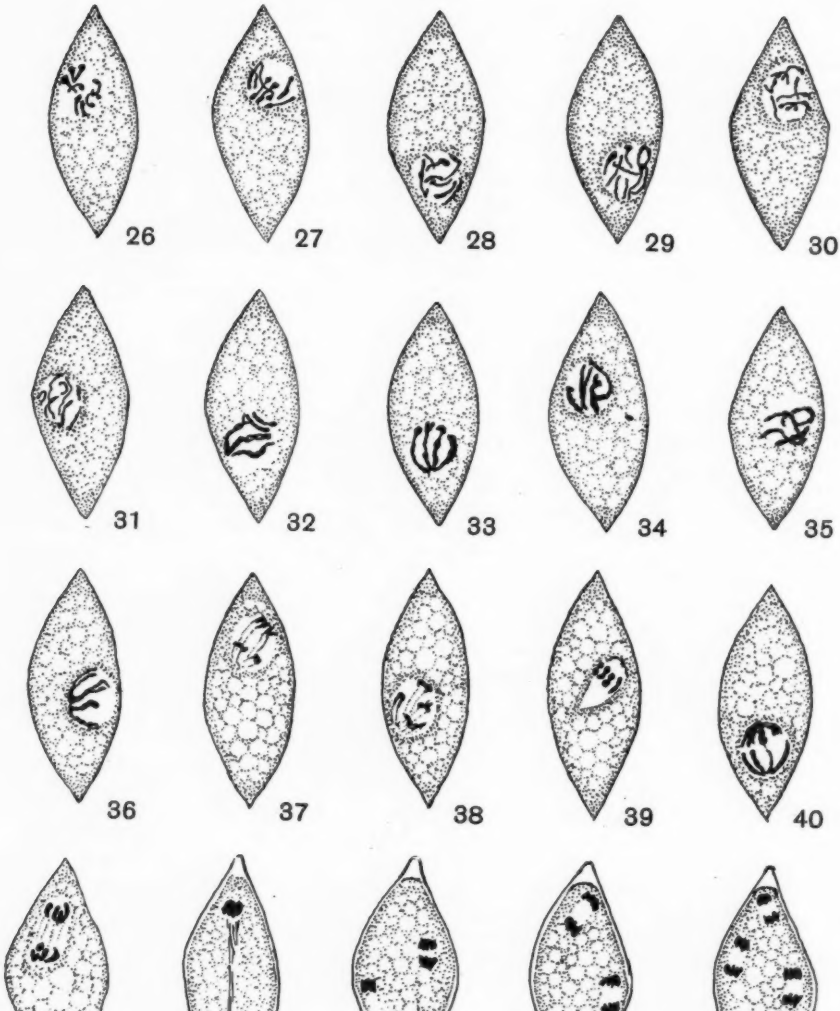
Figs. 16–25. *Apolocystis elongata* n.sp.



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Figs. 26–45. *Apolocystis elongata.*

Nuclear division in the zygote. From sections of seminal vesicle, fixed alcohol Bouin, stained Heidenhain's iron-haematoxylin. Drawn with help of camera lucida (× 2300 approx.).

Figs. 26–42. First nuclear division. 26, 27, eight chromosomes appear in early prophase; 28–31, leptotene; 32–34, zygotene; 35, pachytene; 36, diplotene; 37, 38, late diplotene or diakinesis (?); 39, prometaphase; 40, anaphase; 41, early telophase; 42, late telophase. 43, second nuclear division, anaphase. 44, 45, later nuclear division, anaphase.

metaphase; Fig. 40 anaphase; Fig. 41 early and Fig. 42 late telophase. Up to Fig. 39 Naville might argue that we were dealing with a gregarine that preserved the ancestral habit of pairing its chromosomes before the first zygotic division, although meiosis had really been shifted back to the pregametic stage. But Figs. 40 and 41 show quite clearly that only four chromosomes are going to each pole, thus re-establishing the haploid number demonstrable in the gamonts in Figs. 16–25.

Figs. 43–45 show anaphase in the later, mitotic divisions within the sporocyst. Completion of this highly impermeable envelope made staining and differentiation difficult, and it was not possible to count the chromosomes with complete certainty at these stages. But the number still seems to be about four: certainly it is not eight. In Fig. 14 the sporozoites have been delimited: they are spirally twisted within the sporocyst. Fig. 15 is an oblique cross-section of a mature sporocyst: it passes through the eight sporozoites at nuclear level.

V. SYSTEMATIC POSITION OF THE GREGARINE

Cognetti (1923) created the genus *Apolocystis* with the following diagnosis: 'solitari corpo globoide mancante di asse principale segnato dalla presenza e disposizione di organiti speciale periferici.' To this genus he removed (1925) *Monocystis lumbrici-olidi* Schmidt, along with five other species of *Monocystis*.

According to Hesse (1909), Schmidt, as early as 1854, observed this rounded gregarine covered with processes, in *Lumbricus olidus* Hoffm. (= *Eisenia foetida* Sav.). Hesse himself gave the first full description of the trophozoite as observed by him in the seminal vesicles of *Eisenia foetida*, *Dendrobaena rubida* Sav. and *D. subrubicunda* Eisen. In a general way, his description accords with what we have noted on pp. 66–7; but the measurements are given as only 90–100μ; the trophozoites are stated to be rounded, ovoid or reniform; the short, ectoplasmic processes, 'tantôt fins, tantôt assez épais', are more close-set in the posterior region and sometimes appear only on a polar cap, whereas in our gregarine they are of uniform thickness and quite evenly distributed. We have already referred (p. 67) to the different staining reactions of the karyosomic complex in our gregarine and in the one Hesse observed. On these grounds, therefore, the parasite from the Bristol brandlings cannot be classified as *Apolocystis lumbrici-olidi* (Schmidt). The only other species of *Apolocystis* recorded from brandlings is *A. gigantea*, described by Troisi (1933) from *Helodrilus* (*Eisenia*) *foetida* in the eastern United States.* The trophozoites are stated to be 'usually spherical and without polarity in movement': the diameter of the majority is given as 300–500μ (i.e. about twice the size of the largest we observed in Bristol brandlings): the 'hairs' on the ectoplasm measured 10–15μ in length (which is about three times their length in our gregarine): the sporocysts are also rather larger, the dimensions being given as $19 \times 8{\cdot}6\,\mu$: moreover, as noted above (in footnote on p. 67), the trophozoite nucleus of *Apolocystis gigantea* never has more than one satellite karyosome, and even this is not constantly present.

It is clear that our gregarine is closely related to the foregoing and must be placed in the same genus. But '*Apolocystis*' scarcely describes a sausage-shaped organism, and the name we propose for the new species, *A. elongata*, may seem a contradiction in terms.

The following is the diagnosis of *Apolocystis elongata*:

Systematic position. *Apolocystis elongata* n.sp. (Gregarinida, Monocystidae).

Description. Trophozoites sausage-shaped when extended: measurements up to $220 \times 95\,\mu$: ectoplasmic processes $3\,\mu$ in length and uniformly distributed over the entire surface: nucleus always containing one, and commonly two, satellite karyosomes. Sporocysts $16 \times 8\,\mu$ and without residuum.

Habitat. Seminal vesicles of brandlings, *Eisenia foetida* Sav.

Locality. Bristol, England.

VI. SUMMARY

1. A new monocystid gregarine is reported from the seminal vesicles of brandling worms, *Eisenia foetida* Sav. The morphology and movements of the trophozoite are described, and the stages of gametogony and sporogony are outlined.

2. The chromosome cycle is investigated. The haploid number of chromosomes is four, and the reduction division is shown to be zygotic.

3. The systematic position of the gregarine is discussed, and it is placed in the genus *Apolocystis* as *A. elongata* n.sp.

* *A. minuta* Troisi, described in the same paper, was recorded from three species of *Lumbricus*. It is spheroidal, with diameter of only 40μ, and has no ectoplasmic processes.

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THE ECOLOGY OF THE SHEEP TICK, *IXODES RICINUS* L. DISTRIBUTION OF THE TICK ON HILL PASTURE

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(With 2 Figures in the Text)

CONTENTS

	PAGE
1. Introduction	75
2. Relative population densities on different areas of the pasture	75
(i) On sheep paths (tracks) as compared with the general pasture	75
(ii) On sheep lairs (night resting places) as compared with the general pasture	76
(iii) In relation to altitude	76
3. Distribution on uniform pasture	77
(i) A uniform stretch of hill pasture	77
(ii) Sampling method	77
(iii) Blanket-dragging samples and population density	78
(iv) The time factor	79
(v) The space factor	79
4. Practical conclusions	80
5. Summary	81
References	81

1. INTRODUCTION

A previous paper on northern England (Milne, 1944) deals with the distribution of *Ixodes ricinus* in four counties, part of a county, a group of farms and a single typical hill farm, the detail increasing as the area lessens. The present paper fills in more details for the last-mentioned farm. This 1200 acre Cheviot hill farm, Hethpool, has sheep grazings on two fairly steep hills, one less steep and one gently sloping; and in the floors of two valleys running between these hills. The grazings are rough grass, chiefly white bent (*Nardus stricta*). Bracken has encroached upon approximately 40%. Relevant points from the paper mentioned above are: (i) The thicker the vegetation layer, the denser the tick population; thus thin, medium and thick layers had relative nymphal densities of 1·0, 2·2 and 4·6–6·1 respectively. (ii) The thicker the vegetation layer the thicker the basal mat of decaying vegetable matter; and, within limits, the thicker the mat, the denser the tick population.

2. RELATIVE POPULATION DENSITIES ON DIFFERENT AREAS OF THE PASTURE

(i) *On sheep paths (tracks) as compared with the general pasture*

Nuttall (1911) noted that replete *Boophilus decoloratus* 'rattled like peas on the ground' when a cow was taken from its *warmed* stall into the *cool* air of the courtyard in Cambridge. Observing that cattle and game paths are sometimes particularly infested, he suggests (apparently for ticks in general) that temperature may influence this just as in *B. decoloratus*, since 'it is along them (the paths) that animals like cattle frequently return from pasture in the cool of the evening'.

In hill country, sheep feed on the lower slopes and in the valleys during the day. In the cool of the evening they make for the heights, where they spend the night, always along the same well-defined narrow paths. The infestation of these paths has been compared with the neighbouring pasture.

Observations were made on one typical hill of Hethpool which rises from approximately 400 ft. above sea-level to about 750 ft. at the top. At the bottom is a flat valley floor, then a very steep rise of 20–30 ft. to the edge of a gently sloping plateau and finally the long steep rise to the hilltop. At intervals along the first steep rise, clearly defined paths lead upwards off the valley floor. Observations were confined to between 400 and 600 ft. above sea-level. Between these levels, five or six pairs of drags were evenly spaced out on three occasions (Table 1). Each pair consisted of a 50 yd. drag along the path and a parallel drag 6–10 yd. off to the side in the normal pasture. The path being less than a foot wide, the bordering vegetation to a width of 2 ft. to 2 ft. 6 in. was included on either side by the blanket. In all, seventeen pairs of drags were made on the three paths. The difference in the average nymphs per drag was not significant ($P = 0{\cdot}5$). Hence sheep paths on the hill were no more highly infested than the surrounding vegetation well off the paths, i.e. the general pasture. Of course, conditions are rather different from the

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Table 1

(∞ = nymphs)

Date	Drag	On path ♂♂	On path ♀♀	On path ∞	6–10 yd. off path ♂♂	6–10 yd. off path ♀♀	6–10 yd. off path ∞
13 Apr.	1	1	0	14	1	0	8
	2	0	2	21	0	0	10
	3	0	0	12	0	0	24
	4	0	0	4	0	0	9
	5	0	0	18	0	0	22
27 Apr.	6	0	0	1	0	0	3
	7	0	1	6	1	0	1
	8	0	0	3	0	0	3
	9	0	0	0	0	1	0
	10	0	0	0	0	0	0
	11	0	0	5	1	0	15
5 May	12	0	0	7	0	0	6
	13	0	0	0	0	0	3
	14	0	0	3	0	0	7
	15	0	0	3	0	0	0
	16	0	0	0	0	1	0
	17	0	0	1	0	0	3
Totals	17	1	3	98	3	2	114
Means per drag		—	—	5·8	—	—	6·7

The first five small-scale observations show that very few ticks live on the night lair as compared with the day pasture. This was later confirmed by the large-scale observation no. 6 ($P = < 0{\cdot}001$) showing population densities of nymphs on day pasture and night lair in the approximate ratio 25:1. This ratio would have been greater if drags during low or nil activity at either end of the spring season had been omitted. For some unexplainable reason the differences in the case of males or females are not so marked.

The lower population density of ticks on lairs must be largely due to the vegetation conditions. The lair is invariably composed of fine short herbage on dry ground (thin vegetation layer), the general pasture of coarse, very much taller herbage on damper ground (thick vegetation layer). For example, in observation no. 6, the lair is composed of fine grass about 2 in. long with practically no true mat and fairly open; the general pasture is composed of rough grasses 12–18 in. long with true mat 3·15 in. deep and very dense. The thick layer is very much more suited to tick survival (see Introduction).

Possibly replete ticks do drop off in large numbers on the lairs but most should be unable to breed. Hence the lairing habit of hill sheep probably causes the death of many ticks.

Table 2

Date	Total simultaneous drags on each area	Total ticks in all drags: Night lair area ♂♂	Night lair area ♀♀	Night lair area ∞	Day pasture area ♂♂	Day pasture area ♀♀	Day pasture area ∞	Mean nymphs per drag: Night lair area	Mean nymphs per drag: Day pasture area
(1) 4 Apr.	3	0	0	0	0	0	7	0·00	2·33
(2) 12 Apr.	2	0	0	0	0	0	3	0·00	1·50
(3) 13 Apr.	1	0	0	0	1	0	2	0·00	2·00
(4) 25 Apr.	1	0	0	0	2	4	19	0·00	19·00
(5) 9 June	2	1	0	3	1	2	15	1·50	7·50
(6) 14 Apr.–9 June	66 (3 per day)	3	3	14	5	8	342	0·21	5·18

African cattle or game paths which Nuttall no doubt had in mind. For example, the difference between midday and evening temperatures is less in this country.

(ii) *On sheep lairs (night resting places) as compared with the general pasture*

Hill sheep lie up for the night in recognized sheep lairs, areas of the pasture almost invariably near the hilltop. Since the lairs occupy a very much smaller area than the general pasture grazed by day, we might expect that ticks would be much more numerous on the lairs. Table 2 shows observations on night lairs as compared with day pastures on three hills of the heavily infested Hethpool farm where the activity peak occurs in April or early May (Milne, 1945*a*).

(iii) *In relation to altitude*

The introduction showed that the thinner the vegetation layer the less dense the tick population. On a typical hill the vegetation layer almost invariably becomes thinner and finer, and the ground drier on proceeding uphill. It follows that tick population density should normally decrease with increase in altitude on a hill. Blanket-drag data for three Hethpool hills in Table 3, confirm this point for nymphs. The case is not so clear with males and females but the data is scanty. However, confirmation of both points is seen in subsection (ii) and Table 2 (above), the night lairs being several

hundred feet above the tested areas of the day pastures.

In the above, we purposely chose typical gradients in which the vegetation layer became thinner and the ground drier with increase in height above sea-level. *Small* localized repetitions of lower altitude vegetation-type are, however, occasionally found at higher altitudes due to soil conditions. Examples were studied on hill C (400–1100 ft.) on two occasions (Table 4). These data show that, provided vegetation conditions are suitable, altitude has no limiting effect on nymphal density distribution within the levels studied.

Since in general vegetation conditions become less suitable with increasing altitude on a hill, nymphal population density also becomes smaller. The same explanation should apply to the fact that sometimes the farms at the upper end of a valley are less infested than those at the lower end. (The data might have been fuller but enough has been done to show that distribution according to altitude is, as expected, a function of vegetation conditions.)

3. DISTRIBUTION ON UNIFORM PASTURE

(i) *A uniform stretch of hill pasture*

The vegetation over a hill pasture often varies a good deal (Milne, 1944). A uniform stretch may be defined as a single area having broadly a uniform plant species composition throughout, and a uniform density and thickness of herbage cover throughout. The density and thickness are here judged by the eye only. On uniform stretches we might expect ticks to be more or less uniformly distributed, since no one part should attract the chief distributors (sheep) more than another.

Table 3

Date	Hill	No. of drags	Height of drag above sea-level (in ft.)	Total ticks in all drags ♂♂	♀♀	∞	Mean nymphs per drag
2 Apr.	A	6	500	0	1	9	1·5
	500–1130 ft.	3	900–1100	1	0	0	0·0
12 Apr.		1	500	0	0	9	9·0
		1	800	0	1	2	2·0
25 Apr.*	B	1	400– 500	2	4	19	19·0
	400–750 ft.	1	500– 600	4	0	19	19·0
		1	600– 750	0	0	0	0·0
13 Apr.	C	2	400– 450	2	0	3	1·5
	400–1100 ft.	2	500– 550	1	0	0	0·0
		2	700–1100	0	0	0	0·0
9 June		3	450– 500	1	2	17	5·7
		3	600– 800	0	1	3	1·0
		3	900–1050	1	0	1	0·3

* Blanket dragged downhill between the heights indicated; all other drags are the standard horizontal 50 yd. in length.

Table 4

Date	No. of drags	Height of drags above sea-level (in ft.)	Vegetation layer	Total ticks in all drags ♂♂	♀♀	∞	Mean nymphs per drag
2 May	5	450	Thick	1	2	9	1·80
	3	750	Thick	0	1	11	3·67
9 May	2	650– 900	Thin	0	0	2	1·00
	2	1000–1050	Thin	1	0	1	0·50
	2	800–1000	Medium	1	1	2	1·00
	2	1100	Medium	0	0	4	2·00
	4	500– 600	Thick	1	2	18	4·50
	1	1050	Thick	0	1	14	14·00

(ii) *Sampling method*

Were it possible to estimate tick ground populations by some *direct* method such as that for wireworms which are present up to 2000 per sq.yd. (Salt & Hollick, 1944; Milne, 1943), much experimentation and theorising on *indirect* methods (as in this section) would be avoided. Unfortunately probably no more than twelve nymphs are present per square

yard in the heaviest infestations (see subsection (v) below) so that the labour of examining turves of adequate size would be prohibitive besides having other drawbacks.

The blanketing method for comparing tick populations of plots is indirect and has already been detailed (Milne, 1943). Briefly: (*a*) the nymph count is the indicator; (*b*) sets of two or three 25 or 50 yd. simultaneous drags are made on the plots on not less than 20 days spaced over the tick season; (*c*) a virgin strip of ground is taken for each drag in one season.

Point (*c*) requires some explanation and qualification partly omitted in the paper quoted. Ticks cannot be returned from the blanket to their natural positions on the ground. If destroyed, then subsequent corresponding sets of drags must obviously contain the *same* proportion of previously partly denuded ground. The use of virgin strips of ground for each drag is much simpler. Particular strips to be dragged on any day should, of course, be allotted at random. Unfortunately, no comprehensive system of flagging or pegging has been found proof against sheep and cattle for the duration of a tick season. The less satisfactory method adopted was therefore an orderly progression across each plot, starting at and along corresponding sides and moving in one blanket breadth for each drag. With the virgin strip method, the minimum size of plot required is half an acre. But there is no reason why ground should not be covered twice in the same orderly manner, at right angles the second time, provided all the ticks taken the first time were destroyed. Indeed, this would tend to lessen errors due to lack of random allotment of strip positions. In covering the ground twice over, the minimum size of plot necessary would be a quarter of an acre. This area could be reduced still further by covering the ground more often.

(iii) *Blanket-dragging samples and population density*

In blanket-dragging, the *activity* of nymphs at the moment of dragging is sampled. Fig. 1 shows nymphal activity-density curves for three plots, two on one hill pasture (*L*, bracken; *C*, rough grass) and one on an adjacent hill pasture (*E*, rough grass). Simultaneous sets of two drags, taken on 3 days a week, are shown as averages per drag per week. The bracken plot *L* has a higher activity density than the grass plot *C*, and the latter higher than the grass plot *E*. Aberrations occur on 27 May and 3 and 10 June. Here the rise of *C* and *L* must be due to the blanket riding on the growing bracken and hence picking up fewer ticks (see Milne, 1943). In general, however, Fig. 1 shows that in adjacent areas of similar or different vegetation type, activity-density curves are similar in trends whatever their absolute level. The factors influencing activity must therefore be common to all the plots—most probably they are the meteorological conditions. Hence *simultaneous* drags are necessary for estimating relative activity-

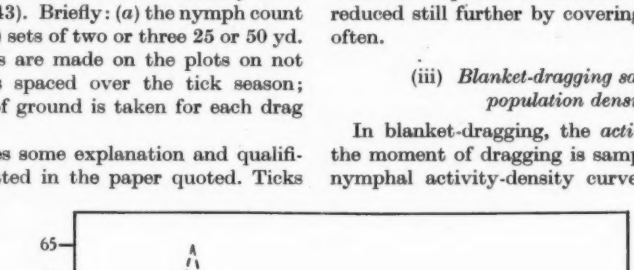

Fig. 1

density on plots. Now, in the present work, activity-density is assumed proportional to ground-population-density. Factors of time and space must be taken into consideration here.

(iv) *The time factor*

Depending on the time at which gorged larvae drop to the ground, and on the average temperature duration for development, one plot might have the majority of its nymphs active at an earlier date in the season than another plot. Such a state of affairs might arise in two fenced fields grazed at different times by sheep over a period of years. On a uniform stretch of hill pasture different age groups of gorged larvae are likely to be similarly distributed by sheep on the ground, yet the above consideration cannot be entirely ignored. The only way to get over this difficulty is to distribute the simultaneous drags evenly over the entire active season.

(v) *The space factor*

Take for example the following set of three parallel contiguous drags (made one after another on a uniform pasture) which yielded totals of 5, 4 and 24 nymphs. This sort of variation is quite frequent (see Milne, 1943, Table 6). It may really be that two of the strips of land had similar ground populations while the third had a much higher population. To clear up this problem, 'exhaustion' dragging was carried out (after the suggestion of J. Allan Campbell (1944) unpublished). Three parallel plots, $50 \times 2 = 100$ sq.yd., *P*, *Q* and *R*, were marked out on a uniform stretch of rough grass on one of the Hethpool hill pastures (191 acres with 150 sheep). The mid-points of the plots were 15 yd. apart. Two drags per day, in opposite directions, were taken on each plot for the number of days shown in Table 5. Each drag covered each plot entirely. The ticks from each drag were killed. From 24 June (end of Table 5) on to November, the totals collected from *P*, *Q* and *R* were 1 nymph, 3 nymphs and 0 ticks respectively. Hence, apart from a week or two at the end of March, the entire active season was covered. Plots *P*, *Q* and *R* yielded totals of 1009, 700 and 715 nymphs respectively. Although the blanket is not a completely efficient collecting instrument (Milne, 1943), and although the sheep may have carried a few ticks away in grazing over the unfenced plots, the frequency of blanketing justifies the idea that the actual populations could not have been very much greater than the totals given. The plots yielded 7–10 nymphs per square yard for the year 1944. Probably the actual population is no more than 12 per square yard on such heavily infested land.

Table 5

Week ending	No. of days of dragging	Plot *P*			Plot *Q*			Plot *R*		
		♂♂	♀♀	∞	♂♂	♀♀	∞	♂♂	♀♀	∞
1 Apr.	6	1	2	56	0	0	19	1	2	42
8	6	3	0	119	3	1	84	2	3	99
15	5	4	2	205	2	1	111	0	6	146
22	6	1	3	151	1	0	82	0	1	63
29	6	0	0	90	1	1	75	2	4	84
6 May	6	2	4	75	1	0	53	0	0	6
13	6	2	4	149	1	2	94	0	2	105
20	6	1	0	33	0	0	29	0	0	21
27	6	0	2	66	1	2	73	1	3	68
3 June	5	0	4	8	0	2	33	1	0	24
10	6	1	2	19	1	0	11	0	0	31
17	6	0	1	12	0	0	20	0	1	13
24	4	0	2	26	0	0	16	0	0	13
Totals	74 = 148 drags per plot	15	26	1009	11	9	700	7	22	715

From an analysis of variance of drags $F = 11{\cdot}23$, with D.F. $= 2$ and 146, hence there is a significant difference among the plot totals of nymphs. The standard error of plot totals is 77·85, therefore plot *P* has a significantly greater nymphal population than either *Q* or *R*, whose populations are not significantly different from each other. (With males and females the results are erratic, owing probably to the relative smallness of the sampling area in relation to their sparser density distribution.) Thus nymphal populations were not uniformly distributed in the chosen 100 sq.yd. sections of uniform pasture. Since the differences are not too great, however, we might expect uniform distribution where larger areas are taken as units for comparison.

Table 5 also shows that the trends of weekly nymphal yields are the same on each plot, and that

new ticks must become active for the first time each week as the season progresses (Milne, 1945*b*).

While *P*, *Q* and *R* were being observed, three other plots with similar activity-density levels and on the same uniform pasture—*C* (rough grass), *L* and *A* (both rough bracken)—were being blanketed by the usual method, i.e. two drags each per day *using a virgin strip of land for each drag*. Drags were made on the average 3 days per week as opposed to 6 days in the case of *P*, *Q* and *R*. Fig. 2 compares the weekly yields per drag for the two methods. Activity-density trends are similar by both methods, again indicating the influence on activity of some factor(s) common to the plots. The higher averages on the virgin stretches, plots *C*, *L* and *A*, are due, of course, to the weekly catch being comprised not only of ticks becoming active that week but also of ticks which have been active for some time (Milne, 1945*b*). The latter will be much less in the case of plots *P*, *Q* and *R*, owing to the frequent and continuous blanketing of the *same* stretches.

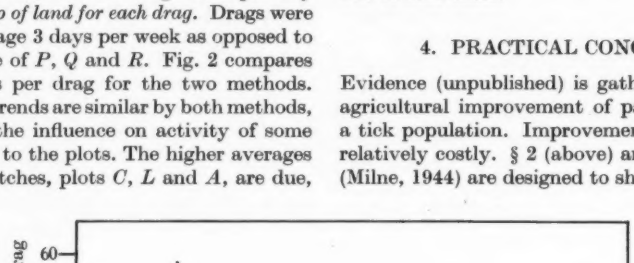

Fig. 2

Data given above show that the numbers of ticks on 100 sq. yd. units of uniform pasture may differ significantly. What then is the minimum area unit on which populations might be expected to be similar? For areas approaching 1 acre and over 1 acre, the 'exhaustion' method is impracticable. The variation is such that too many 100 sq.yd. strips would be required (to estimate absolute populations on each plot) for the labour to be possible. Hence we return to the sampling method outlined in subsection (ii) above where relative population densities are estimated from samples of activity-density. In Table 6, simultaneous sets of two or three virgin 50 yd. drags were taken on neighbouring untreated plots within large areas of uniform Hethpool pasture until the entire plots were covered once by blanket. The first three experiments (Table 6) each provide a comparison of a pair of neighbouring plots, the fourth experiment provides six comparisons, i.e. a total of nine pairs. In only four cases out of the nine was there no significant difference between the members of the pair. Hence Table 6 shows that neighbouring plots of area 1 acre, within uniform stretches of pasture, which were expected to have similar relative population densities, did not in five out of nine cases.

4. PRACTICAL CONCLUSIONS

Evidence (unpublished) is gathering to show that agricultural improvement of pastures may reduce a tick population. Improvement of hill-grazings is relatively costly. § 2 (above) and a previous paper (Milne, 1944) are designed to show which areas of a grazing carry the heaviest populations, i.e. would give the best return for pasture treatment. Sheep paths and lairs cover a very small fraction of the total grazing. But since lairs have a very much lower population than, and tracks a similar population to, the general pasture, they are not worth singling out for anti-tick treatment. However, by far the majority of a tick population is confined to the lower slopes and valleys of a *typical* hill grazing. Hence pasture improvement can most profitably be applied in these areas. Where the hill is atypical, improvement should be confined to the 'roughest' (thickest vegetation) areas.

In pasture-improvement plot-testing, the ideal method is to estimate relative population densities of both control and experimental plots *before* treatment of the latter is commenced. This is not always possible. Sometimes the 'experimental' treatments have already been carried out by the farmer himself, or he is unwilling to wait another 6-12 months. The attempt to estimate the minimum area-unit, of a uniform stretch of pasture (see § 3), on which populations might be expected to be similar, is designed to meet this difficulty. In five out of nine cases, pairs of adjacent plots within uniform stretches of

hill pasture had significantly different populations. Hence non-replicated experiments should be preceded by population estimations of proposed experimental and control plots. This is specially the case with large hill pastures stocked at one sheep to one or more acres where the sheep have a considerable choice of ground and hence may distribute ticks unevenly (space factor). It is less important in enclosed uniform fields with denser stocking and consequently greater likelihood of even distribution of gorged ticks by sheep. Where the stretch of uniform pasture is large enough for sufficient replications, a random block or latin square arrangement should obviate the necessity for prior estimations of population density.

becomes thinner, and nymphal density decreases, with altitude. The case is not so clear with males and females. Provided vegetation conditions are suitable (i.e. sufficiently thick), altitude has no limiting effect on nymphal density distribution within the levels studied. Tick density distribution is therefore a function of vegetation conditions, the latter usually being affected by altitude.

Distribution of ticks on apparently uniform areas of vegetation is studied. Sampling methods are discussed. Probably no more than twelve nymphs per square yard of ground are present on the average in heavily infested pasture. The numbers of nymphs per 100 sq. yd. differed significantly. The number per acre (approx.) also differed significantly in rather more than half the cases observed. Larger area-units within uniform stretches of pasture probably carry similar numbers of ticks.

Practical applications of the findings to the study of tick control are indicated.

Table 6

Date	Plot type	Size of plot in yards	Mean nymphs per drag	Standard error of difference between two means	D.F.
(1) 23 Apr.–24 May	(i) Bracken	60 × 50	17·90		
	(ii) Bracken	60 × 50	22·83	3·05	9
(2) 17 Apr.–17 June	(i) Grass	120 × 50	3·00		
	(ii) Grass	120 × 50	3·33	0·61	19
(3) 3 Apr.–10 June	(i) Bracken	120 × 50	25·58		
	(ii) Bracken	120 × 50	20·25	2·41	29
(4) 3 Apr.–10 June	(i) Grass	120 × 50	3·62		
	(ii) Grass	120 × 50	4·62		
	(iii) Grass	120 × 50	1·82		
	(iv) Grass	120 × 50	5·38	0·68	87

5. SUMMARY

Further details of tick distribution (*Ixodes ricinus* L.) on the pastures of a typical hill farm are given.

Sheep paths (tracks) and their borders have tick populations similar in density to that of the general pasture.

Sheep lairs (night resting places) have significantly lower nymphal densities than the general pasture. With males and females, though the difference is in the same direction, it is less well marked.

On the typical hill, the vegetation layer generally

Thanks are due to Dr V. B. Wigglesworth, F.R.S., Director, Agricultural Research Council Unit of Insect Physiology; Dr A. E. Cameron, Edinburgh University; and Captain the Hon. Claud Lambton, on whose farm the work was carried out.

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THE BIOLOGY OF *ORNITHODORUS DELANOEI ACINUS* WHITTICK, 1938

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INTRODUCTION

Four females and a pre-adult nymph of this tick (see Whittick, 1938) were sent here from the British Museum in April 1941. They were a part of the original type material of *Acinus* Whittick. They had arrived at the Museum two years previously from British Somaliland, and were now found to be still alive. The ticks were put into a constant temperature room at 25° C., with the humidity approximately at 50% saturation. The five ticks successfully engorged on a rabbit's ear, and the nymph moulted to become a female. Several batches of eggs were laid from time to time, but they all shrivelled and were sterile. Eventually, towards the end of September 1942, several larvae hatched from a batch of eggs laid a week or two earlier, and these were used for a study of metamorphosis in this tick.

METHOD

All nymphal stages and adults were allowed to take their blood meals on the shaved pinna of a rabbit after the method of Jobling (1924–5) for *Ornithodorus moubata*. This method was convenient, since these stages take only about a quarter of an hour to feed.

A different method had to be employed for feeding the larvae because they were found to attach to the host for several days. Feeding on guinea-pigs over water (the method often used for *Ixodes*) was not favoured as the number of specimens of this little-known species which were available was small, and nothing was known of their survival or capabilities in water. Therefore an area of guinea-pig's flank, about 5 cm. across, was closely clipped, and a screw-topped, bottomless, metal capsule fixed over it with an 'Elastoplast' bandage girding the whole trunk. The clipped area could thus be inspected with very little trouble, while any ticks which detached themselves were prevented from escaping. No ventilation was found to be necessary. Guinea-pig hair grows rapidly, and every 3 or 4 days it was necessary to cut the hair in the arena with small scissors so that the larvae could easily be observed. At the end of experiments the bandage was removed by cutting the hair to which it was adhering. (For this method I am indebted to Dr E. T. Burtt, of the Insect Physiology Unit, Agricultural Research Council, who has used it for *Ixodes ricinus*.)

When not feeding, the ticks were kept in small dishes with sand for the larger stages, or filter-paper for the smaller stages. The temperature was regulated at 30° C., and the relative humidity, though not strictly controlled, was about 50%.

The egg. Although the female is much larger than that of *Ornithodorus moubata*, the eggs of the two species are of approximately the same size. The developmental period of only one small batch of eggs was studied, half of which proved sterile. The sterility was not surprising since these females had not been in company with a male for at least 3 years (see Robinson, 1942). The average period of development was 22 days, varying from 21 to 25. These figures are based on observations on eighteen eggs.

The larva. The period between emergence and willingness to attach to the guinea-pig was 9·5 days varying from 8 to 11 days. Observations were taken on only four larvae.

The period of engorgement was long, and lasted from 9 to 16 days, averaging 12 days. The feeding room was at 20° C., and did not vary more than a degree above or below the average. The larva clings very closely to the host's skin in which it often makes a slight depression to fit its body. All larvae voided white faecal matter during the period of their attachment. For the first few days of engorgement, only the creases in the larval skin flattened out. It was only in the last day or two on the host that the bulk of the blood was taken in, when the cuticle stretched enormously to accommodate the meal. Only one out of twenty-three larvae was seen to have secreted coxal fluid on the host, and this was probably because it had been confined in the capsule some time after it had detached itself from the guinea-pig. Often when larvae detached themselves from the host they had a little collar of the scarf skin left round the base of the mouthparts. The bite seemed to give the guinea-pig no inconvenience; there was no swelling and no bruising. Healing was very quick. Sometimes a larva would die when attached, and then an elongated clot would form round the proboscis and this and the overlying skin would be shed with the tick attached.

The average period before the engorged larvae moulted was 9·3 days, varying from 7 to 13. Fifteen were observed. The figures given here for the developmental periods of the larvae of the subspecies

acinus do not differ very much from the observations of Roubaud & Colas-Belcour (1936) on those of the type species which were also fed on a guinea-pig.

The nymphal stages. Compared with the larvae, the nymphs take a very short time to feed—a matter of minutes instead of days. Although they are very sluggish creatures when they are not on their host, they soon become quite active on a rabbit's ear before they pierce the skin to feed. Nymphs are able to feed 2 days after emergence from the larval or previous nymphal stage. The nymphs rarely secreted coxal fluid during engorgement on the host. A minute or two after leaving, they secreted a large quantity in a very short time (about 25 % by weight of the blood imbibed), and the stretched cuticle become dented on the dorsal aspect. Later the dents arranged themselves along the symmetrically placed discal furrows. The faeces of the nymphs were blackish, indicating the presence of decomposed blood. Nymphs larger than the first stage were found to be willing to feed more than once before moulting. This is no doubt necessary owing to the habit of excreting the coxal fluid after feeding. Thus *O. moubata* always starts excreting quite early in the process of engorgement, and can consequently take in more blood cells than would be contained in its own capacity of unchanged blood, owing to the simultaneous voiding of some of the watery fraction of the meal. On the other hand, *O. delanoei acinus* takes its full capacity of blood, and excretes some of the watery fraction later, thus leaving room for another meal.

The tick-bites did not appear to cause any irritation to rabbit or guinea-pig, but bruises were visible on the guinea-pig for a couple of days after bites were received. The effect of the bite on the human subject was not tried, because of the possibility of the ticks being infected with spirochaetes.

The periods between feeding and moulting of the nymphs were as follows:

Stage	Average period in days	Range	No. of ticks	Adults produced
First	18	13– 31	23	—
Second	27	19– 38	20	—
Third	61	20–126	18	—
Fourth	100	52–327	15	7 ♂ 2 ♀
Fifth	91	44–120	6	1 ♂ 2 ♀
Sixth	92·5	92– 93	2	2 ♀

Roubaud & Colas-Belcour (1936) record observations on one individual, apparently a third-stage nymph, of the type species which took 212 days to moult at 25° C. It would not feed before another 449 days. It was then kept at 28° C., and metamorphosed to a female after 385 days. The authors say that this long time might have been due to the experimental host being a guinea-pig, and that the ticks might develop more quickly on the porcupine, the natural host. They do not say how often this tick was given the opportunity to feed.

Adults. Seven males emerged from fourth-stage nymphs and one from a fifth-stage nymph. Two were kept under observation, and these succeeded in mating only after a blood-meal which they took 6 and 15 days respectively after emergence. They were each kept in a small dish with a female apiece known to be capable of mating. This subspecies is thus different from *O. moubata*, males and females of which are capable of mating a few hours after emergence.

Females were derived from fourth, fifth and sixth nymphal stages, and each of these instars produced two females. The females resembled the males in their failure to mate before a blood-meal. The time before newly emerged females would take a meal was very variable. Five were observed, and the interval averaged 86 days, but varied from 15 to 188. Only one female was observed between mating and oviposition. She laid eggs 58 days after copulation. Roubaud & Colas-Belcour (1936) mention one female of the type species which fed about a year after emergence, and mated shortly afterwards. Eggs were laid about 9 months after this.

It must be mentioned that the adults of the *acinus* subspecies just discussed were at most two-thirds the linear dimensions of the original specimens lent by the British Museum. It may be that the optimum developmental temperature is lower than that used in these experiments (30° C.). At a lower temperature the developmental times would be longer, and it is possible that the derived adults would be larger.

SUMMARY

The life history of *Ornithodorus delanoei acinus* under laboratory conditions at 30° C. may be summarized thus:

Stage	Average period in days	Range	No. of ticks	Adults produced
Egg	22	21– 25	18	—
Larval	31	24– 40	15	—
1st nymphal	20	15– 33	23	—
2nd nymphal	29	21– 40	20	—
3rd nymphal	63	22–128	18	—
4th nymphal	102	54–329	15	7 ♂ 2 ♀
5th nymphal	93	44–120	6	1 ♂ 2 ♀
6th nymphal	92·5	92– 93	2	2 ♀
Emergence of ♀ to meal	86	15–188	5	—
Mating to oviposition	58	—	1	—

(These figures include the interval between emergence and willingness to feed, and the period of engorgement of the larvae.)

Thanks are due to Prof. P. A. Buxton, F.R.S., in whose department the work was done, and to Dr A. D. Lees of the Insect Physiology Unit, Agricultural Research Council, who made final observations on some of the later nymphal stages and adults.

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EXPERIMENTS UPON THE FEEDING OF *AËDES AEGYPTI* THROUGH ANIMAL MEMBRANES WITH A VIEW TO APPLYING THIS METHOD TO THE CHEMOTHERAPY OF MALARIA

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(With 2 Figures in the Text)

CONTENTS

	PAGE
I. Introduction	85
II. Strains of *Aëdes aegypti* and *Plasmodium gallinaceum* used	85
III. The method of feeding *Aëdes aegypti* through a membrane:	
(1) Type of membrane	86
(2) Method of feeding	86
IV. The effect of age upon feeding in *Aëdes aegypti*:	
(1) Feeding upon chickens	86
(2) Feeding through membranes	87
V. The effect of a heat gradient upon the proportion of *Aëdes aegypti* feeding through a membrane	87
VI. *Aëdes aegypti*. The effect of the application of saliva to the outer surface of the membrane on the proportion gorging on blood	88
VII. The use of the membrane technique in a study of the feeding reactions of *Aëdes aegypti*	89
(1) Chick plasma	90
(2) Washed corpuscles	90
(3) Haemolysed blood	90
(4) A comparison of the results of feeding *A. aegypti* through a membrane and from open drops	91
(5) Discussion of the results from feeding experiments	94
VIII. The infection of *Aëdes aegypti* with *Plasmodium gallinaceum* through animal membranes	95
IX. A method for obtaining viable sporozoites of *Plasmodium gallinaceum* by feeding infected *Aëdes aegypti* through animal membranes	97
X. Summary	100
References	100

I. INTRODUCTION

The primary object of our experiments upon the feeding of *Aëdes aegypti* through membranes was, as stated in a previous communication (Bishop & Gilchrist, 1944), to obtain sporozoites free from all trace of glandular tissue in order to test the action of drugs directly upon them. Hitherto the only method of obtaining sporozoites was by dissection of the salivary glands, and by this method it is difficult to be certain that all the sporozoites are freed from cellular tissue and equally exposed to the admixed drugs.

Gordon (1922) had succeeded in making *Stegomyia* (=*Aëdes*) *calopus* feed through the skin of a bat, though they never fed to repletion even on whole blood. Woke (1937) had induced *Aëdes aegypti* to feed through a rat-skin membrane upon whole blood or certain fractions of it, and Yoeli (1938) had caused *Anopheles elutus* to gorge through prepared rabbit-skin membranes upon blood containing gametocytes of *Plasmodium falciparum*, and obtained infection of the mosquitoes.

We decided to find out if *Aëdes aegypti* infected with *Plasmodium gallinaceum* would, when fed through a membrane, eject viable sporozoites, and if so, if they would be ejected in numbers sufficiently numerous for experimental purposes.

II. STRAINS OF *AËDES AEGYPTI* AND *PLASMODIUM GALLINACEUM* USED

Three strains of *Aëdes aegypti* have been used in the experiments described herein. One strain, referred to as the B strain, was that used by James & Tate (1938) as a vector for *Plasmodium gallinaceum* and was given to them by Prof. E. Brumpt, Faculté de Médecine, Paris: the second strain, referred to as the G strain, was the gift of Prof. R. M. Gordon of the Liverpool School of Tropical Medicine and was used as a vector for *P. gallinaceum* by Lumsden & Bertram (1940); and the third, the C strain, was the gift of Dr Coatney of the National Institute of Health, U.S.A.

The strain of *P. gallinaceum* was obtained from Prof. E. Brumpt in 1937.

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III. THE METHOD OF FEEDING *AËDES AEGYPTI* THROUGH A MEMBRANE

(1) *Type of membrane*

The first problem in setting out to obtain viable sporozoites by feeding infected mosquitoes through a membrane was to discover the most satisfactory type of membrane, and the optimum conditions for inducing *Aëdes aegypti* to feed through it. In his experiments Woke (1937) used the skins of week-old rats. Yoeli (1938) took the skin from a rabbit's ear and, after soaking it in 96% alcohol, washed it and dried it stretched over a glass cylinder. In our experience the skin of young chickens not more than 2–3 weeks old has produced the best membranes. Whereas the skin of young rats, whether fresh or fixed in alcohol, tears easily and does not adhere to the glass cylinder over which it is stretched, chicken skin is extremely strong and adherent when prepared by soaking in absolute alcohol for 30 min. or longer, followed by prolonged washing in water. It is then dried, stretched over one end of the glass cylinder (6 × 2·5 cm.) to be used in the feeding experiments, and held in place by a rubber band. The skin dries to form a thin, parchment-like membrane which adheres so firmly to the outer surface of the glass cylinder that it does not come loose even if soaked in water, and is absolutely watertight. Such membranes can be stored on their cylinders for long periods, and can be used several times if they are washed after use and resoaked in absolute alcohol before being dried.

Cellophane membranes were tried, but found to be unsuitable.

(2) *Method of feeding*

The mosquitoes to be fed are placed in glass cylinders approximately 12 × 9 cm. Both ends of the cylinders are covered with mosquito-netting held in place with strong rubber bands. In the net covering the top of the cylinder a small hole is made which can be closed with a cotton-wool plug. Through this hole the mosquitoes are introduced. To provide the damp atmosphere necessary to successful gorging the mosquito-cylinders are stood over Petri dishes containing damp filter paper.

Experiments have proved (p. 87) that the highest proportions of *A. aegypti* feed through a membrane when the temperature of the blood at the commencement of the feeding experiment is approximately 40–42°C. We have devised, therefore, a water jacket surrounding the membrane cylinder, to prevent the temperature of the blood from falling rapidly. The water jacket is made partly of rubber and partly of glass (Fig. 1), the rubber base being held in place round the membrane cylinder and the outer cylinder of the water jacket by strong rubber bands. The initial temperature of the water jacket should be approximately 48°C., as heat is lost in warming up the apparatus. The blood, at 40–42°C., is poured into the membrane cylinder when the temperature of the water jacket has reached that temperature. The apparatus is then stood on the top of the mosquito cylinder so that the surface of the membrane rests upon the net.

Feeding is performed in the dark at 28°C. and, unless otherwise stated, the mosquitoes are allowed 30 min. in which to feed.

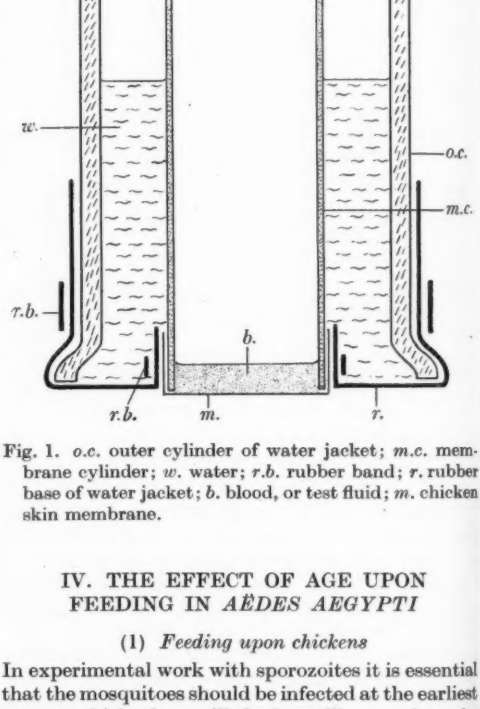

Fig. 1. *o.c.* outer cylinder of water jacket; *m.c.* membrane cylinder; *w.* water; *r.b.* rubber band; *r.* rubber base of water jacket; *b.* blood, or test fluid; *m.* chicken skin membrane.

IV. THE EFFECT OF AGE UPON FEEDING IN *AËDES AEGYPTI*

(1) *Feeding upon chickens*

In experimental work with sporozoites it is essential that the mosquitoes should be infected at the earliest age at which they will feed readily, so that the greatest number possible should survive until the salivary glands become infected. In order to discover the earliest age for successful feeding under the conditions available to us, we made a series of experiments with female mosquitoes of different age groups increasing in intervals of 24 hr. from emergence, with a minimum of 0–24 hr. and a maximum of 72–96 hr. In each series of experiments all the mosquitoes used were derived from the same batch of eggs and were bred under the same conditions. The mosquitoes in all the groups of the same series, therefore, were similar except in age. From the time of emergence until the first blood meal

they were given no food, but were allowed access to water.

In feeding mosquitoes upon chickens the mosquitoes were applied to the breast of the chicken in a biting box. The experiments were performed in the dark at 28°C., in a moist atmosphere, and the mosquitoes were allowed 30 min. in which to feed. In assessing the numbers which had fed, only those which had gorged fully were counted.

The results (Table 1) show that in both the B and C strains there is a gradual increase in the proportion of mosquitoes gorging up to a maximum at 72–96 hr. but when the percentages of all the age groups are compared (Table 2), the proportions gorging are found to increase with increase in age as in the experiments with chickens. This increase in proportions gorging with increase in age was found in all three strains of *A. aegypti*.

Whereas in feeding *A. aegypti* directly on a chicken the earliest age groups in which a high proportion of mosquitoes gorged was 48–72 hr., when fed through a membrane 72–96 hr. from emergence was the earliest age at which gorging was consistently high.

Table 1. Aëdes aegypti. *The effect of age upon the proportions gorging upon a chicken*

	B strain			C strain		
Age of *Aëdes* in hours	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
0–24	3	8/126	6	1	0/51	0
24–48	3	56/135	41	1	26/73	36
48–72	3	104/137	76	1	89/94	95
72–96	1	23/29	79	1	74/79	94

Table 2. Aëdes aegypti. *The effect of age upon the proportions gorging through membranes*

	B strain			C strain			G strain		
Age of *Aëdes* in hours	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
0– 24	4	13/258	5	3	11/209	5	1	0/47	0
24– 48	4	89/302	29	2	22/152	14	1	3/46	7
48– 72	4	151/301	50	2	59/159	37	1	2/42	5
72– 96	2	105/193	54	2	124/170	73	1	10/33	30
96–120	—	—	—	3	190/284	67	—	—	—
120–144	—	—	—	2	132/186	71	—	—	—

The 48–72 hr. group was the youngest in which a high proportion of the mosquitoes gorged. Above the 72–96 hr. group little consistent increase in the proportions gorging has been found.

Seaton & Lumsden (1941), working under carefully controlled conditions of temperature and humidity, found that when virgin female *A. aegypti* were offered blood meals from the human forearm the proportions gorging increased through the age groups from 0–24 hr. to 72–96 and 96–120 hr.

When infecting *A. aegypti* with *Plasmodium gallinaceum* by feeding on infected chickens we therefore, whenever possible, use mosquitoes 48–96 hr. old.

(2) *Feeding through membranes*

The experiments were similar to those with chickens, with the exception that the mosquitoes were offered blood at 40–42°C. through a membrane according to the method described above. Much wider variation was observed in the same age groups in the different series of experiments with mosquitoes fed through membranes than on chickens,

V. THE EFFECT OF A HEAT GRADIENT UPON THE PROPORTION OF *AËDES AEGYPTI* FEEDING THROUGH A MEMBRANE

Preliminary experiments showed that when *A. aegypti* were offered blood through a membrane the proportions of mosquitoes which gorged was much greater if the blood was warmed to 40–42°C. than if its temperature was that of the environment. The question therefore arose whether the mosquitoes were attracted to warmed blood by the presence of a heat gradient, or whether the heat given off from the blood provided a microclimate of optimum temperature for gorging.

In experiments designed to elucidate this question mosquitoes of the same age derived from the same batch of eggs and bred under similar conditions were collected into five groups (with the exception of one experiment in which only four groups were used). Although all the groups of mosquitoes in each particular experiment were of the same age, the age of the mosquitoes in different experiments

varied from 72–96 to 120–144 hr. No food was given to the mosquitoes between the time of their emergence and of the experimental feeding. The experiments were performed in the dark, at 28°C., in a saturated atmosphere.

Fresh heparinized chick blood was used for feeding the mosquitoes.

Using water-jacketed membrane cylinders containing fresh heparinized chick blood on top of cylinders of female *A. aegypti* in the manner previously described, a series of experiments was set up as follows:

A. Temperature of environment 24°C. Temperature of blood 24°C. Temperature gradient 0.

B. Temperature of environment 28°C. Temperature of blood 28°C. Temperature gradient 0.

28°C. is a more favourable temperature to gorging than 24°C. and thus more than compensated for the slightly smaller gradient.

That in those experiments where the blood was heated to 42°C. it was the heat gradient which accounted for the high proportions of mosquitoes which gorged, and not the presence of a warm microclimate immediately below the membrane, is proved by the fact that when the temperature of the blood and the environment were both high (37°C.) the rate of gorging was much lower than when a heat gradient was present.

Since all differences other than that of temperature were eliminated in these experiments it can be concluded that a heat gradient is an important factor in attracting *A. aegypti* to its food.

Table 3. Aëdes aegypti. *The effect of a heat gradient upon the proportions gorging*

Temperature of blood ...	24°C.			28°C.			37°C.		
Temperature of environment ...	24°C.			28°C.			37°C.		
	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
	6	26/430	6·0	6	35/439	8·0	5	74/393	19

Temperature of blood ...	42°C.					
Temperature of environment ...	24°C.			28°C.		
	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
	6	224/436	51	6	285/403	71

C. Temperature of environment 37°C. Temperature of blood 37°C. Temperature gradient 0.

D. Temperature of environment 24°C. Temperature of blood 42°C. Temperature gradient 24–42°C.

E. Temperature of environment 28°C. Temperature of blood 42°C. Temperature gradient 28–42°C.

It will be seen from Table 3 that in those cylinders where the temperature of the blood was the same as that of the environment the proportion of mosquitoes which gorged was low, though it increased with the rise in temperature of the environment. But in those cylinders in which there was a gradient between the temperature of the blood and that of the environment the proportion which gorged was high. In the cylinders where this heat gradient was greatest (24–42°C.) the proportion of mosquitoes which gorged was not so high as in those where the gradient was 28–42°C. The explanation may be that

VI. *AËDES AEGYPTI*. THE EFFECT OF THE APPLICATION OF SALIVA TO THE OUTER SURFACE OF THE MEMBRANE ON THE PROPORTION GORGING ON BLOOD

In feeding *Aëdes aegypti* upon fowls, Lumsden & Bertram (1940) found that if mosquitoes showed a reluctance to feed they could often be induced to do so if saliva were applied to the skin of the fowl. In the light of these observations it seemed possible that an application of saliva to the outer side of the membrane might increase the proportion of *A. aegypti* feeding through it. Preliminary experiments (Bishop & Gilchrist, 1944) seemed to indicate that an increase in the proportion of mosquitoes feeding did occur when saliva was applied to the membrane. Further experiments, however, in which females of the same age group and strain were offered fresh heparinized chick blood from the same

source under identical conditions, with the exception that in one case the outer surface of the membrane was dry whereas in the other it was moistened with saliva (Table 4), proved that saliva had little effect upon the proportion of mosquitoes feeding through the membranes.

Table 4. Aëdes aegypti. *The effect of moistened (saliva) membrane upon proportions gorging*

Membrane moistened with saliva			Dry membrane		
No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
9	270/474	57	9	239/469	51

VII. THE USE OF THE MEMBRANE TECHNIQUE IN A STUDY OF THE FEEDING REACTIONS OF *AËDES AEGYPTI*

In studying the feeding mechanism of mosquitoes the technique used by earlier workers (Fülleborn, 1908; Kadletz & Kusmina, 1929; MacGregor, 1930, 1931; Roy & Gosh, 1940) was that of the capillary tube. By this method the fluid on which the mosquito is to be fed is put into the tube and this is slipped over the mouthparts of the mosquito. MacGregor (1931) maintained that when this technique was used on mosquitoes with the proboscis unsheathed the results obtained were comparable with those from true biting; but Roy & Gosh (1940) considered that such results were irregular and not comparable with natural feeding. It seemed to us possible that a technique such as is offered by the use of prepared membranes, in which the act of feeding involves piercing and is similar to natural biting, and in which the factors involved can be carefully controlled, might yield useful information on the feeding reactions of mosquitoes.

That the destination of different kinds of foods in the internal organs of the mosquito was not the same had been noted by many workers. Nuttall & Shipley (1903) were the first to show that when *Anopheles maculipennis* were fed on food containing sugar, and killed immediately after the feed the three diverticula were filled with the fluid. Wright (1924) confirmed this, and found that after a blood meal little of this fluid was found in the diverticula, the greater part being in the stomach. According to Kadletz & Kusmina (1929), who used the capillary tube technique, blood was directed to the crop, stomach and diverticula whereas sugary fluids were directed to the crop and diverticula.

MacGregor (1930, 1931) described two methods of feeding in mosquitoes; true biting and 'discontinuous suction'. According to him, when mosquitoes feed by biting through the skin of the host the destination of the blood imbibed is the stomach, whereas that of water, fruit saps or other sugar-containing fluids ingested by discontinuous suction is the diverticula. He believed that mosquitoes exercise a 'selective and voluntary control' in regard both to the aspiration and destination of the fluids ingested. Using the capillary tube technique with unsheathed proboscis, he found that mosquitoes sometimes would imbibe blood when they had refused honey, and sometimes the reverse reaction would occur. When blood containing a trace of honey was offered to *Aëdes aegypti* by the capillary technique it was despatched to the diverticula, instead of passing directly to the stomach as would be the case with pure blood.

Roy & Gosh (1940) compared the results of feeding mosquitoes upon honey, glucose or citrated blood imbibed from wet cotton-wool and by forcible feeding by the capillary tube technique. In *A. aegypti* the sugary fluids, when given by the former method, were directed mainly to the diverticula which were fully distended though a trace might be found in the stomach, whereas citrated blood was directed primarily to the stomach, though a trace was found in the diverticula. When these foods were fed forcibly to *A. aegypti* the results were inconsistent. Blood ingested naturally by biting was directed to the stomach and diverticula, all of which were fully distended.

Yoeli & Mer (1938) fed *Anopheles elutus* upon solutions of haemoglobin, suspensions of blood cells, and serum through animal membranes 'in order to prevent their penetration into the diverticulum of the mosquitoes, which occurs regularly when the anophelines do not feed on an animal'. They give no data to support this assertion.

We decided to use the technique of membrane feeding in studying the reactions of *A. aegypti* to different types of food and, by comparing the results of feeding similar fluids through a membrane and in open drops, to obtain more light upon the problem of whether the method of feeding or the nature of the food determines its destination in the mosquito.

In each of the feeding experiments the mosquitoes used were females of the same strain and age group. Approximately equal numbers were put into each of two biting cylinders. On top of one cylinder a membrane apparatus was stood containing the test fluid at 40–42° C., and on the other, as a control, a similar apparatus, also at 40–42° C., containing heparinized whole blood freshly drawn from a chick. Feeding was effected in the dark, at 28° C., in a moist atmosphere, and the time allowed was 30 min.

Since some of the fluids tested were colourless, and could not therefore be detected easily in the alimentary canal of the mosquitoes, it was first

ascertained whether the addition of cochineal to whole blood had any effect upon the proportion of *A. aegypti* which gorged. Five drops of an aqueous solution of cochineal were added to 3–5 c.c. of the test fluids. A series of experiments was performed, according to the method described above, in which the test fluid was heparinized whole chick blood coloured with cochineal. The addition of cochineal had no inhibitive effect upon feeding, in fact in the small series (Table 5) of experiments which was performed the mean percentage of mosquitoes feeding on blood and cochineal was higher than that of those feeding on blood alone.

All the mosquitoes which ingested blood, or blood and cochineal, were fully gorged, with the exception of two (Table 5) in which the stomach was only partly filled. No trace of blood was found in the diverticula of any of the mosquitoes.

Table 5. Aëdes aegypti. *Comparison of feeding reactions to whole blood and whole blood + cochineal*

	Whole blood		Whole blood + cochineal		% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Whole blood + cochineal
9	41/87 = 47%	39 + S 2 (+) S	51/91 = 56%	51 + S	45	56

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

Table 6. Aëdes aegypti. *Comparison of feeding reactions to whole blood and plasma*

	Whole blood		Plasma		% fully gorged	
No. of experiments	Proportion containing fluid	Distribution of fluid and amount ingested	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Plasma
4	84/126 = 67%	84 + S	13/124 = 10%	13 (+) S	67	0

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

(1) *Chick plasma*

A comparison was next made of the feeding reactions of *A. aegypti* to the two main components of blood, in order to discover whether plasma and corpuscles were equally attractive to the mosquitoes.

Plasma was prepared by centrifuging fresh, heparinized chick blood and removing the clear, supernatant fluid. A few drops of cochineal solution were added to the plasma and to the whole blood to be used as a control. The temperature of the fluids offered was 40–42° C. Although a normal proportion of the control mosquitoes gorged fully upon the whole blood, it appeared by direct examination that none of those offered plasma had ingested any, but upon dissection a trace was found in the stomachs of 13 out of 42 mosquitoes in one experiment (Table 6). In none of the mosquitoes was either blood or plasma found in the diverticula. All the mosquitoes which ingested whole blood were fully gorged.

These results suggest that some factor is missing from plasma which induces or enables *A. aegypti* females to gorge upon whole blood. The feeding reactions of *A. aegypti* to washed corpuscles therefore were studied.

(2) *Washed corpuscles*

Corpuscles were prepared by centrifuging fresh, heparinized chick blood and removing the plasma. The corpuscles were washed twice in citrate saline solution and suspended in this fluid, the proportion of saline to corpuscles being equal to that of the plasma removed. Heparinized whole chick blood was used as a control. In all four experiments (Table 7) some of the mosquitoes which were offered washed corpuscles ingested them, though the mean percentage was lower than of those which ingested whole blood. Whereas those mosquitoes which imbibed plasma ingested only a trace, all those which ingested washed corpuscles gorged fully. The ingested food, irrespective of whether it was corpuscles alone or whole blood, was present in the stomach and never in the diverticula.

Since mosquitoes gorged fully upon washed corpuscles whereas they either failed to feed upon plasma or ingested only a trace of it, it seemed probable that the corpuscles contained some substance attractive to the mosquitoes. If this were so the rupturing of the corpuscles by haemolysis might liberate more of the attractive substance and so induce a greater proportion of mosquitoes to gorge.

(3) *Haemolysed blood*

Haemolysed blood was prepared by the rapid freezing and thawing of heparinized chick blood.

The viscid solution of haemoglobin in plasma was freed from the stroma by centrifuging. The proportion of mosquitoes which ingested haemolysed blood was smaller than of the controls which ingested whole blood (Table 8). Whereas, however, almost all the mosquitoes which ingested normal blood gorged fully, of those which ingested haemolysed blood the number which gorged fully was smaller than of those which contained a mere trace of fluid visible only on dissection. Since the viscosity of blood haemolysed by this method is very great it seemed possible that this physical property might account for the small proportion of mosquitoes which gorged diluted none of the mosquitoes ingested blood (Table 8).

It seemed possible that the greater facility of ingestion by *A. aegypti* of whole as compared with haemolysed blood might be explained by the mechanical factor of the presence in whole blood of particulate matter in the form of corpuscles. In order to study the mechanical effect upon gorging of the addition of small particles to haemolysed blood, a suspension of rice starch granules in a solution of haemoglobin in plasma was offered in a membrane to *A. aegypti*, but none of the mosquitoes ingested it.

Table 7. Aëdes aegypti. *Comparison of feeding reactions to washed corpuscles and whole blood*

	Whole blood		Washed corpuscles suspended in citrate saline		% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Proportion containing corpuscles	Distribution of corpuscles and amount ingested	Whole blood	Washed corpuscles
4	88/136 = 65%	86 + S 2 (+) S	50/130 = 38%	50 + S	63	38

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

Table 8. Aëdes aegypti. *Comparison of feeding reactions to haemolysed and whole blood*

	Whole blood		Haemolysed blood				% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Fluids	No. of experiments	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Haemolysed blood
5	157/227 = 69%	156 + S 1 (+) S	Haemoglobin in plasma	6	178/341 = 52%	52 + S 126 (+) S	69	15
			Diluted 1 : 2	2	7/72 = 10%	7 (+) S		
			Diluted 1 : 4	1	23/73 = 32%	11 + S 12 (+) S		
4	59/121 = 49%	57 + S 2 (+) S	Haemoglobin in water	4	47/144 = 33%	38 + S 9 (+) S	47	26
			Haemoglobin in water diluted 1 : 2	1	0/24 = 0%	0	—	0

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

to repletion. Dilution of the fluid with water, however (Table 8), caused a decrease, not an increase, in the proportion of mosquitoes which imbibed the fluid, and of these few gorged to repletion.

As a further test of the influence of viscosity upon feeding, a solution of haemoglobin in water was prepared by haemolysing washed corpuscles in water and centrifuging off the stroma. The proportion of water to corpuscles was the same as that of the plasma removed. The proportion of mosquitoes which fed on this fluid was smaller than in the controls (Table 8), but of those which ingested the fluid a greater proportion gorged fully than upon haemoglobin in plasma. When the solution was

In none of the mosquitoes, whether fed upon heparinized whole blood, haemolysed blood, washed corpuscles or plasma, was any trace of the fluid found in the diverticula; the stomach alone contained it.

(4) *A comparison of the results of feeding* A. aegypti *through a membrane and from open drops*

In order to discover whether the method of feeding or the nature of the food determines its destination to stomach or diverticula, the results were compared when female *A. aegypti* of the same age group were offered similar fluids (*a*) through a membrane and (*b*) from a drop on a glass slide. The

initial temperature of the fluid in the membrane was 40–42°C., and since a water jacket at the same temperature was used the fall in temperature was slow; whereas although the temperature of the fluid in the drops in the first place was 42°C., it fell rapidly to that of the environment (28°C.).

When *heparinized blood* was offered in drops (Table 9) a much smaller proportion of *A. aegypti* fed upon it than when it was offered through a membrane. The only difference in conditions was that of the temperature of the fluids and the method by which the food was offered. All the mosquitoes found in the diverticula of mosquitoes which imbibed it through a membrane.

Of the small proportion (3/66) of *A. aegypti* which ingested haemoglobin in water from drops, 2 had a trace in the stomach and the third a trace in both stomach and diverticula. None of the mosquitoes ingested it through a membrane.

The results with *plasma* were similar whether it was offered in drops or through a membrane (Table 9). Only a small proportion of mosquitoes ingested a trace of it and this was found in the stomach.

Table 9. Aëdes aegypti. *Comparison of feeding reactions to fluids in drops or through membranes*

		Drops			Membrane		
			Distribution of fluid in mosquitoes			Distribution of fluid in mosquitoes	
No. of experiments	Test fluid	Proportion containing fluid	S	D	Proportion containing fluid	S	D
3	Blood	18/108	16 +	0	74/147	74 +	0
			2 +	2 (+)			
1	Haemoglobin in plasma	0/46	0	0	26/53	8 +	8 +
						18 (+)	0
1	Haemoglobin in water	3/66	1 (+)	1 (+)	0/61	0	0
			2 (+)	0			
1	Plasma	7/19	7 (+)	0	7/22	7 (+)	0
			(Total number of mosquitoes dissected 73)				
4	Glucose	139/207	56 (+)	56 +	20/202	12 (+)	0
			0	17 +		0	2 (+)
						3 (+)	3 +
						3 (+)	3 (+)
			(Total number of mosquitoes dissected 42)				
2	Honey	80/93	5 (+)	5 +	0/91	0	0
			0	37 +			
1	Washed corpuscles in 8% glucose	8/26	0	2 +	6/17	6 +	0
			3 (+)	3 +			
			3 +	3 (+)			
1	Blood + honey	37/41	37 (+)	37 +	5/37	5 (+)	0

Unless otherwise stated all mosquitoes in each experiment were dissected.

\+ = fully gorged. S = stomach.
(+) = small quantity or trace of fluid ingested. D = diverticulum.

which imbibed blood from the drops gorged fully and, with the exception of 2 which had a trace of blood in the ventral diverticulum, blood was found only in the stomach. The stomachs of the mosquitoes which fed through the membrane were distended with blood and the diverticula empty.

When *haemoglobin in plasma* was offered in drops (Table 9) none of the mosquitoes ingested it, whereas 26 imbibed it through a membrane, 18 containing a trace of fluid in the stomach and the remaining 8 having both stomach and diverticula fully distended. This was the only occasion on which blood or any component part of blood was

With few exceptions, therefore, the destination of blood, whether whole or haemolysed, and of plasma is the stomach, and is the same whether the method of intake involves true biting or discontinuous suction.

Since fruit juices and other sugar-containing fluids normally are imbibed by discontinuous suction and not by 'biting', and since their destination when thus ingested is the diverticula, the results of feeding female *A. aegypti* of the same age groups upon honey or a saturated solution of glucose by discontinuous suction (i.e. from open drops) and by biting (i.e. through a membrane) were compared.

Both honey and glucose were coloured with cochineal and warmed to 40–42°C.

When saturated glucose solution was offered in drops a high proportion of mosquitoes gorged fully upon it, with the exception of one experiment when gorging was poor (Table 9). The diverticula of these mosquitoes were fully distended with the fluid and the stomach of most individuals contained a trace. In marked contrast to these results few or none of the mosquitoes offered glucose through a membrane imbibed it, and then almost without exception in small quantities only. The glucose was found in either stomach or diverticula or in both.

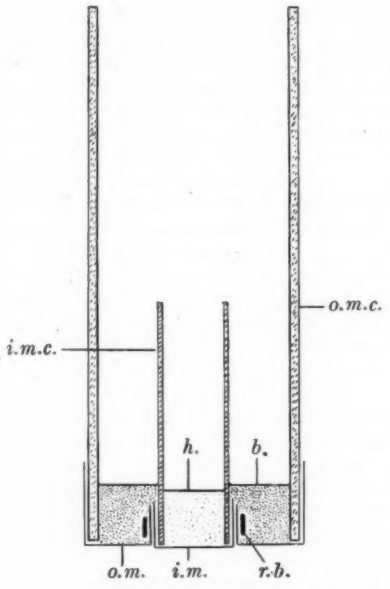

Fig. 2. *o.m.c.* outer membrane cylinder; *i.m.c.* inner membrane cylinder; *b.* blood; *r.b.* rubber band; *h.* honey; *o.m.* outer membrane; *i.m.* inner membrane.

None of the mosquitoes imbibed honey through a membrane though the proportion which gorged on it from drops was high (Table 9). In these the diverticula were fully distended with honey and, in a few mosquitoes on dissection, a trace was found in the stomach also.

These experiments demonstrate that whereas *A. aegypti* will gorge freely on honey or glucose solution from drops, either they are unaware of these fluids when presented in a membrane, or find difficulty in aspirating them. It is difficult to believe that these solutions are less palatable when offered by this method.

In order to compare the reactions of the same mosquitoes under identical conditions to carbohydrates and blood we prepared two concentric membrane tubes (Fig. 2) with honey and cochineal in the inner tube and blood in the outer tube. The outer tube was surrounded by a water jacket at 40–42°C. and the apparatus stood on a cylinder of female *A. aegypti* of the same age group at 28°C. Twenty-three of the mosquitoes gorged fully on the blood which was directed to the stomach only. No trace of honey was found in the stomach or diverticula of these mosquitoes, or in those which did not imbibe blood. This experiment was repeated using a saturated solution of glucose coloured with cochineal in the outer membrane and blood in the inner membrane. Twenty-four out of 52 mosquitoes gorged fully in 30 min. and these contained blood in the stomach only. No trace of glucose was found either in the stomach or diverticula of these mosquitoes or of those which failed to ingest blood. When this experiment was repeated with glucose in the inner membrane and blood in the outer, 23 out of 54 mosquitoes gorged fully. One of these mosquitoes contained only glucose. The ventral diverticulum was fully distended with the fluid, but no trace of it was present in the stomach. Twenty-two mosquitoes contained blood in the stomach only, no trace of glucose being found in stomach or diverticulum, and the remaining 31 contained neither blood nor glucose.

As it seemed possible that an olfactory factor might be responsible for this selection of foods the following experiment was performed. Two membrane tubes were arranged one inside the other, the outer one containing saturated glucose solution coloured with cochineal and the inner one whole blood. The outer surface of the membrane containing the blood came into close juxtaposition to the glucose solution which was only 1 mm. or so in depth. It was hoped that if mosquitoes were attracted to blood by some odour which was given out through the membrane this might impregnate the glucose solution and render it attractive to the mosquitoes. But of the 39 mosquitoes in the biting cylinder none gorged on the glucose.

The effect of mixing blood or its components with glucose solution or honey was next studied. A small proportion of *A. aegypti* (Table 9) imbibed a suspension of corpuscles in 8% glucose from drops. In 5 out of 8 the ventral diverticulum was fully distended, 3 of these having traces of the suspension in the stomach also, whereas in the remaining 3 the stomach was fully distended and a trace was found in the ventral diverticulum. In all 6 mosquitoes which imbibed the suspension through a membrane the stomachs were fully distended, no trace being found in the diverticulum. A high proportion of mosquitoes imbibed a mixture of blood and honey from drops, and in these the diverticula were fully

distended with the fluid and the stomach contained a trace; but when a similar mixture was offered through a membrane the proportion which imbibed it was small and only a trace was ingested which was located in the stomach.

A comparison of the feeding reactions of *A. aegypti* of the same age group to plasma, honey and a mixture of these substances when offered through a membrane gave some anomalous results (Table 10).

Table 10. Aëdes aegypti. *Comparison of feeding reactions to honey, plasma, and a honey-plasma mixture fed through membranes*

No. of experiments	Test fluid	Proportion containing fluid	Distribution of fluid in mosquitoes dissected	
			S	D
4	Honey	0/174	0	0
2	Plasma	11/83	11 (+)	0
1	Honey + water 1 : 1	1/48	0	1 (+)
			(Total number of mosquitoes dissected 62)	
3	Honey + plasma 1 : 1	57/157	27 (+)	27 +
1	Honey + plasma 5 : 1	6/32	0	6 +

Unless otherwise stated all mosquitoes in each experiment were dissected.

\+ = fully gorged.
(+) = small quantity or trace of fluid ingested.
S = stomach.
D = diverticulum.

Whereas none of the mosquitoes ingested honey and few ingested plasma and then only in small quantities, in two of the experiments the proportion of mosquitoes which ingested a mixture of these fluids was high and in each case the mosquitoes were fully gorged. In one experiment all the mosquitoes were dissected and in 21 out of 56 the diverticula were fully distended with the fluid, and the stomachs contained a trace of it. In each of the other experiments only 3 of the mosquitoes which had gorged fully were dissected, but the distribution of the fluid in the mosquitoes' organs was similar to that in the previous experiment. This increase in the proportion of mosquitoes which gorged on the mixture of honey and plasma as compared with either of the component parts was not due merely to the dilution of the honey, for when the results of experiments with similar dilutions of honey and water, and honey and plasma, were compared it was found that whereas only 1 out of 48 mosquitoes gorged on the honey and water, 21 out of 56 gorged on the plasma and honey.

(5) *Discussion of the results from feeding experiments*

From an analysis of these experiments upon the feeding reactions of *A. aegypti* the following deductions appear to be warrantable. Whole blood, when offered through a membrane, is ingested more consistently than either plasma, haemolysed blood, or washed corpuscles suspended in saline, and the mosquitoes almost invariably gorge fully upon it. *A. aegypti* gorge fully on washed corpuscles also, but the probability of gorging is less than with whole blood. A much smaller and more variable proportion of mosquitoes ingest haemolysed blood and of these many imbibe only a small quantity of the fluid. Plasma is seldom ingested, and then only in small quantities. The destination of all these fluids when ingested through a membrane is the stomach, with the exception that in a few mosquitoes fed upon haemolysed blood the ventral diverticulum also was fully distended.

The proportion of *A. aegypti* which ingest whole blood or haemolysed blood from drops is much smaller than the proportion ingesting them through membranes. The proportion ingesting plasma is equally poor from drops or membranes. When ingested from drops the destination of all these fluids is primarily the stomach, as is the case when they are ingested through membranes, though a trace of the fluid may be found in the diverticula also.

In comparing the results of other workers using a membrane technique we find that Gordon (1922) induced *A. aegypti* to feed upon serum, washed red cells, and whole blood, either from open watch glasses or through the skin of a bat, but by neither method did the mosquitoes feed to repletion on any of these fluids. Woke (1937) fed *A. aegypti* through membranes upon red cells suspended in Ringer's solution, plasma, serum, and rabbit haemoglobin in water, but he gives no data of the proportion of mosquitoes which fed nor of the amount of fluid imbibed. As he was studying the effect of food upon egg production it is to be concluded that the mosquitoes ingested sufficient of the fluid for it to be visible without dissection.

An analysis of the results of feeding *A. aegypti* upon glucose or honey shows that their reactions to these fluids are the reverse of those with blood. The proportion of mosquitoes imbibing glucose or honey from drops is high whereas few imbibe glucose and none honey through a membrane. When imbibed from drops the primary destination of these fluids is the ventral diverticulum which is usually fully distended, but traces of the fluids may be found in the stomach also. Glucose imbibed through a membrane may be present in the diverticula or stomach or both; but of the few mosquitoes which

gorged fully it was the diverticula, not the stomach, which were fully distended.

From these results, therefore, it can be concluded that it is the nature of the food, not the method of feeding, which determines its destination whether into the stomach or diverticula of the mosquito.

The results from feeding experiments with mixtures of blood or plasma and honey or glucose are variable, but as a whole they agree with those which MacGregor (1931) obtained with the capillary tube technique, i.e. in the majority of mosquitoes fed upon such mixtures, whether from drops or through membranes, the ventral diverticulum is fully distended, and the stomach contains a small quantity of the fluid.

Two problems have arisen in the course of these feeding experiments for which there are as yet no explanations: first, why *A. aegypti* should gorge freely on glucose or honey from drops and yet seldom imbibe the former and never the latter fluid through a membrane; and secondly, why they should gorge through a membrane upon a mixture of plasma and honey whereas they do not imbibe honey and seldom plasma, and then only in small quantities, when offered to them separately under identical conditions. The absence of a sustained heat gradient may account for the smaller proportion of *A. aegypti* which gorges from drops of blood as compared with blood through a membrane; but this explanation will not hold for the glucose or honey experiments since the mosquitoes fail to gorge on these fluids through a membrane in the presence of a well-sustained heat gradient, whereas they gorge freely upon them from drops in which the heat gradient is not sustained. Also, when the same mosquitoes are offered a choice of blood or honey, or blood and glucose, under identical conditions (i.e. through concentric membranes, p. 93) the blood is ingested and the honey or glucose neglected. It has been observed in a few instances that *A. aegypti* is able to ingest glucose through a membrane, whilst the ingestion of a mixture of plasma and honey has been observed in many mosquitoes. Both these fluids were directed into the diverticula as in discontinuous feeding. There appears therefore to be no mechanical difficulty in imbibing through a membrane fluids which are destined to the diverticula. The results of the feeding experiments with these fluids suggest that the factor or factors which attract *A. aegypti* to feed upon glucose or honey from drops do not operate through a membrane.

Fülleborn's (1908, 1932) contention based upon experiments with the capillary tube technique, that in *Anopheles maculipennis* aspiration of fluids once begun is automatic and continues until the stomach is fully distended, is not confirmed by our observations upon feeding *Aëdes aegypti* through membranes. These mosquitoes never gorged fully upon plasma and infrequently upon haemolysed blood. They were able to stop aspirating a fluid when only a trace had been ingested.

VIII. THE INFECTION OF *AËDES AEGYPTI* WITH *PLASMODIUM GALLINACEUM* THROUGH ANIMAL MEMBRANES

Yoeli (1938) first proved that mosquitoes (*Anopheles elutus*) could be infected with the malaria parasite by feeding them through a membrane upon defibrinated blood containing crescents of *Plasmodium falciparum*. He found that the blood was not only infective when freshly drawn, but remained so when kept for 5 hr. at 23–27°C., or for 72 hr. in an ice box.*

In the following infection experiments with *P. gallinaceum* the Brumpt strain of *Aëdes aegypti* was used almost exclusively. The rate and intensity of infection in this strain proved very variable even when mosquitoes of the same age group were fed directly upon infected chickens whose blood contained a high proportion of gametocytes of both sexes. A high rate of exflagellation was not a guarantee that mosquitoes gorged upon the chick from which the blood was drawn would become well infected. The proportion of mosquitoes infected and the intensity of their infection may both be low when allowed to gorge on such birds, or the attempt to infect may fail completely.

In the experimental infection of *Aëdes aegypti* with *Plasmodium gallinaceum* by feeding through a membrane, heparinized blood containing a high proportion of gametocytes was used immediately after it had been drawn from the chick. The blood in the membrane apparatus was warmed to 40–42°C. and the mosquitoes were fed in the dark at 28°C., 30 min. being allowed for feeding. A small sample of the blood was kept under observation in order to see whether exflagellation occurred and, if so, after what period of time it began. The mosquitoes which gorged were kept 4–5 days at 28°C. after which their stomachs were examined for oocysts. *Aëdes aegypti* which had gorged in this manner upon blood containing gametocytes of *Plasmodium gallinaceum* became infected (Table 11), and the infections developed normally. Sporozoites were found in the salivary glands and proved to be infective when injected into a chick.

The rate and intensity of infection in mosquitoes

* After our preliminary communication in *Nature*, Prof. R. M. Gordon informed me, in a personal communication, that he and Dr S. Bertram had been working on somewhat similar lines in 1939, and had infected mosquitoes, reared under sterile conditions, with *Plasmodium gallinaceum* by allowing them to gorge on sterile blood through sterile membranes made of sausage skin. A. B.

fed through a membrane on infected blood was compared with that of mosquitoes of the same age group fed directly upon the infected chick from which the blood was drawn. In these experiments one batch of mosquitoes was fed directly upon the chick at 28°C. in the dark, and the second batch was fed through a membrane, under similar conditions of temperature and humidity, upon blood drawn immediately after the first batch had gorged. The gametocytes, therefore, were at a similar stage of development in both experiments.

Whilst in some experiments (Table 11, Exps. 3–5) the rate of infection obtained by the two methods was approximately the same, in others the number of mosquitoes infected when fed through a membrane was lower than when fed directly on a chicken. In many of the mosquitoes which had gorged through a membrane, the number of cysts on the stomach was as great as on those which gorged directly on a chicken.

Table 11. Aëdes aegypti. *Rate and intensity of infection with oocysts of* Plasmodium gallinaceum *when fed (a) through a membrane, (b) on a chicken*

	(*a*) Through membranes		(*b*) On chickens	
	Proportion of *A. aegypti* infected	No. of cysts per stomach	Proportion of *A. aegypti* infected	No. of cysts per stomach
1	3/9	>50, 3, >50	—	—
2	4/7	16, 3, 5, 1	—	—
3	10/20	13, 4, 30, 7, >50, 2, 9, 11, 7, 5	6/13	3, 6, 18, 20, 7, 3
4	3/5	3, 1, 6	5/7	1, 13, 1, 11, 10
5	4/8	1, 1, 2, 3	3/7	1, 1, >50
6	15/20	1, 4, 13, 8, 2, 12, 2, 38, 4, 3, 7, 12, 22, 5, >50	8/8	10, 13, 10, 12, 14, 17, >50, >50
7	1/16	1	4/7	1, 3, 11, 1
8	1/16	2	4/8	3, 4, 2, 19
9	5/13	3, 2, >50, >50, >50	8/8	28, 2, 5, 2, 50, >50, >50, >50
10	1/9	9	4/8	15, 7, 9, 1
11	11/12	1, 1, 1, 2, 30, 4, 2, 17, 5, 47, 35	—	—
12	9/12	1, 3, 8, 16, 1, 4, 2, 1, 10	—	—
13	8/12	2, 3, 1, 1, 1, 5, 1, 5	—	—

In all the experiments recorded in Table 11 in which *Aëdes aegypti* were fed through a membrane, exflagellation was observed in the blood. The length of time before exflagellation began, and the number of male gametocytes exflagellating per field varied widely in different samples of blood. In some experiments exflagellation began before the mosquitoes had access to the blood in the membrane, in others it did not begin until about halfway through the feeding experiment, and in others was not seen until after the membrane apparatus had been removed from the mosquito cylinder. Observations were made in order to discover whether the occurrence of exflagellation in the blood during or before the feeding of the mosquitoes had an inhibitory effect upon the rate of infection; or whether blood in which exflagellation had ceased before it was ingested remained infective. In Exp. 4, Table 11, exflagellation had started before the mosquitoes had access to the blood, and had ceased before the membrane apparatus was removed from them, but infection occurred. In Exp. 6 though no exflagellation was seen until 10 min. after the blood was offered to the mosquitoes, many exflagellating parasites were seen between this period and the end of the feeding experiment; yet the rate and intensity of infection in the mosquitoes which had ingested the blood was relatively high. It was possible, however, that the majority of the mosquitoes infected had gorged before exflagellation became frequent.

The rate and intensity of infection in mosquitoes which had gorged (*a*) before exflagellation could be seen in the blood, (*b*) whilst it was plentiful, and (*c*) after it could no longer be found, were compared. Freshly drawn, heparinized blood containing a high proportion of gametocytes, but in which no exflagellating forms could be seen, was placed in a membrane apparatus upon a cylinder of *A. aegypti* for 10 min. At the time of its removal a few exflagellating forms were found in the blood. Eleven out of 12 of the mosquitoes gorged during the 10 min. became infected (Table 11, Exp. 11) and the intensity of infection was relatively high. The same sample of blood was placed upon a second cylinder of mosquitoes of the same age group. The number of exflagellating forms present was very great during the whole period of 25 min. that the mosquitoes had access to the blood. Nine out of 12 mosquitoes became infected during this period

(Table 11, Exp. 12) and the intensity of infection was moderate. The same sample of blood was kept at +4°C. for 20 hr. after which it was warmed to 40°C. and examined for exflagellation, but none was found either then or at subsequent examinations. Eight out of 12 (Table 11, Exp. 13) of the mosquitoes of the same age group as in the two previous experiments became infected when gorged through a membrane upon the blood, though the intensity of infection was low.

From these observations it can be concluded that though exflagellation may be very frequent at the time of the ingestion of blood by mosquitoes it does not prevent their infection, and blood in which exflagellation has been frequent but has ceased remains infective. It is possible that fertilization of the female gametes had taken place before the blood was ingested by the mosquito, and that the zygotes remained viable. No attempt has been made in the experiments described herein to discover the proportion of male gametocytes undergoing exflagellation. It might therefore be suggested that infection takes place by means of male gametocytes which do not exflagellate until ingested by the mosquitoes; but in Exps. 6 and 12 the numbers of exflagellating parasites was so great that it seemed improbable that there were many mature male gametocytes which were not exflagellating.

IX. A METHOD FOR OBTAINING VIABLE SPOROZOITES OF *PLASMODIUM GALLINACEUM* BY FEEDING INFECTED *AËDES AEGYPTI* THROUGH ANIMAL MEMBRANES

Little is known as yet of the sensitivity of sporozoites *in vitro* to antimalarial drugs, though much evidence has been amassed of the greater resistance to the drugs of sporozoite induced malaria infections of man than of blood-inoculated malaria. It was observed by Yorke & MacFie (1924), in the treatment of general paralysis of the insane, that blood-inoculated infections of malaria and sporozoite-induced infections differed in their response to quinine. The inefficacy of the drug upon sporozoite-induced infections, unless administered for 10–14 days after infection, was attributed to its lack of action upon the sporozoites themselves. A similar lack of response in sporozoite-induced infections to prophylactic treatment with antimalarial drugs in doses highly effective upon blood-inoculated infections was first observed in avian malaria by Russell & Nono (1932) and Tate & Vincent (1933) with plasmoquine, and Kikuth & Giovannola (1933) and Tate & Vincent (1934) with atebrin. These observations have been confirmed by many other workers.

The problem of the nature of this resistance of sporozoite-induced infections of malaria to antimalarial drugs has been complicated by the more recent discovery, in avian malaria, of an exo-erythrocytic cycle of development. Evidence now indicates that in avian malaria, and by analogy probably in human malaria, the sporozoites on entry into the body of the host undergo a phase of development in the tissues (cryptozoites of Huff, Coulston & Cantrell (1943), primary tissue phase of Davey (1944)) before entering erythrocytes. A true causal prophylactic might, therefore, act on the sporozoites, or the primary tissue phase, or on both. A complete knowledge of the mode of action of any antimalarial drug which had been proved to eradicate sporozoite-induced infections in experimental animals would involve a study of its action upon sporozoites *in vitro* and upon the primary tissue phase in culture. By such *in vitro* methods it would be possible to compare the action of a drug upon the sporozoites and primary tissue phase of the species of malaria used in the experimental animal (e.g. *Plasmodium gallinaceum* in chicks) with its action upon the sporozoites and primary tissue phase of any of the species of *Plasmodium* of man. In order to approach such a study it is essential that a method should be devised whereby sporozoites can be obtained free from gland tissue and bacterial contaminants. The method in general use whereby sporozoites are obtained for injection is by dissecting out the infected glands from the mosquito and teasing them apart or grinding them up. Such a method is laborious, and though suitable for infection experiments is unsuitable for the study either of the direct action of drugs upon sporozoites or for tissue culture, for it is impossible to ensure that all the sporozoites are free from traces of tissue, and bacterial contaminants may be present.

Having devised a method whereby *Aëdes aegypti* would gorge readily upon heparinized chick blood, we decided to see whether this technique might be applied successfully to the collection of sporozoites. It appeared to us that success or failure would depend upon the stage in the process of biting at which infected mosquitoes ejected sporozoites. MacGregor (1931) saw no definite sign of salivation when mosquitoes were fed upon freshly drawn blood in capillary tubes, and he considered it probable that saliva was ejected only in the process of incision and not during the act of drawing up the blood. He considered that its function was probably that of a lubricant during actual incision. Gordon & Lumsden (1939) state that 'fluid, presumably salivary secretion, is injected into the tissues at various stages of penetration', but they also noted in one case that it was ejected directly into a capillary.

If, as MacGregor believed, the saliva is merely a lubricant and ejected only during penetration then it seemed unlikely that sporozoites would be ejected

into the blood in the membrane tube when infected mosquitoes gorged; but if saliva is ejected into the capillaries during aspiration then sporozoites should be ejected into the blood when infected mosquitoes gorge through a membrane.

The blood used in feeding infected mosquitoes through a membrane was taken from uninfected chicks preferably not more than 4–5 weeks old. Whereas young chicks infected with *Plasmodium gallinaceum* frequently die a few days after the infection becomes patent, either from the intensity of the infection in the red blood corpuscles, or from the occlusion of the capillaries of the brain by exo-erythrocytic parasites, older birds may survive the acute stage of the infection and lapse into a chronic state, though they may die later from exo-erythrocytic parasites in the brain. Since older birds thus show some degree of immunity to infection with *P. gallinaceum* their blood was not used for the collection of sporozoites.

quitoes which had been infected 8–12 days were most frequently used.

The mosquitoes were allowed 30 min. at 28° C. in which to feed upon the blood through the membrane. The blood was then pipetted out of the membrane cylinder, the inner surface of the membrane being washed with a few drops of saline and the washings added to the blood. The blood was shaken well in order that the sporozoites should be evenly distributed, and injected intravenously into chicks, each chick receiving 0·5 c.c. The birds were examined for the appearance of parasites in the peripheral blood from the 5th day onwards.

In each infection experiment chicks of the same age group were used, the ages ranging from 3 to 18 days. Owing to wartime difficulties it has not been possible to keep to one breed, but no difference in infectivity was found amongst the breeds used.

The proportion of *A. aegypti* which had fed through the membrane was counted. In assessing these numbers it did not appear to be necessary to dissect the mosquitoes which had not gorged, as experience in feeding experiments (pp. 89–93) had shown that, almost invariably, when offered whole blood either they gorged fully or not at all. It was improbable, therefore, that mosquitoes which did not appear to the naked eye to contain blood had ingested any. As in the early stages of the work it was of interest to compare the approximate number of infected mosquitoes used in each experiment with the number of chicks infected from them, the period of incubation, and the intensity of the resulting infections, the salivary glands of some, or all, of the mosquitoes which had fed were examined (Table 12).

Table 12. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Incubation period in chicks inoculated intravenously with sporozoites ejected through a membrane*

	Mosquitoes			Chicks	
	Proportion gorged	No. examined	No. infected	Proportion infected	Day of appearance of parasites in peripheral blood
1	49/84 = 58%	9	8	3/3	7, 7, 7
2	35/57 = 61%	10	6	3/3	6, 6, 6
3	45/72 = 63%	5	5	6/6	7, 7, 7, 7, 7, 7
4	15/32 = 47%	15	5	2/2	6
					Died on 5th day. Exo-erythrocytic forms found in liver and spleen
5	21/41 = 51%	7	7	3/3	6, 6, 6
6	18/110 = 16%	18	11	2/5	7, 10
7	21/52 = 40%	8	4	7/7	6, 7, 6, 7, 7, 6, 7

0·5 c.c. of the blood to be used in the membrane was injected intravenously into a clean chick, in order to prove that no accidental infection of the blood had occurred. These control chicks have invariably remained uninfected.

The heparinized chick blood was put into the membrane apparatus at 40–42° C. according to the usual method, and stood upon the cylinder of infected mosquitoes. In each experiment each chicken was inoculated with 0·5 c.c. blood, and the amount used in the membrane cylinder varied from 1·5 to 4 c.c. according to the number of mosquitoes available and the number of chicks to be inoculated.

It is essential that the *Aëdes aegypti* used for the collection of sporozoites should be well infected. In order to ensure this a few mosquitoes from each batch were examined for oocysts on the 4th or 5th day after being fed on an infected chicken, and only batches in which 50% or more of the sample examined was infected were retained for use. Mos-

The incubation period in chicks injected intravenously with 0·5 c.c. of blood containing sporozoites was usually 6–7 days (Table 12) which was similar to that in chicks of the same age group bitten by 1–4 infected mosquitoes (Table 13). In only one experiment (Table 12, Exp. 6) did some of the chicks

Table 13. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Incubation period in chicks infected directly by mosquito bite*

No. of infected *Aëdes* gorged on chicken	Intensity of infection of salivary glands	Day of appearance of parasites in peripheral blood of chicken
1	++++	7th
1	++++	Negative
1	++	7th
1	++++	7th
2	++++ ++	7th
2	++++ +++	8th
2	++++ ++++	6th
2	+++ ++	6th
3	++++ ++++ +++	7th
3	+++ ++ +	6th
3	++++ +++ +	6th
4	++++ ++++ +++ +++	7th

\+ glands lightly infected with sporozoites.
++ glands moderately infected with sporozoites.
+++ glands heavily infected with sporozoites.
++++ glands very heavily infected with sporozoites.

before mature schizonts in mosquito-induced infections of *Plasmodium gallinaceum* in fowls after suppressive treatment with quinine. They found that gametocytes which had attained, or almost attained, their full dimensions, were clearly recognizable before the first cycle of erythrocytic schizogony was completed, and conclude that their only source was the exo-erythrocytic parasites.

The course of the infection following intravenous injection of sporozoites collected by the membrane technique was similar in intensity and duration to that resulting from the bite of infected mosquitoes. In young chickens death frequently occurred on the 2nd–5th day after the infection became patent, and exo-erythrocytic parasites were found at autopsy in the brain and internal organs. In Table 12, Exp. 3 five out of six of the chicks died a few hours after uninucleate parasites had first appeared in the peripheral blood, and the capillaries of the brain were found to be occluded by enormous exo-erythrocytic parasites. Presumably these birds received a very heavy dose of sporozoites. In one bird (Table 12, Expt. 4) which died accidentally on the 5th day, before parasites had appeared in the peripheral blood, exo-erythrocytic forms were found in the liver and spleen.

Attempts to demonstrate sporozoites in smears made from the blood in the membrane failed, but the bulk of the blood was relatively very great. An attempt therefore was made to see if mosquitoes would be attracted to and gorge from a much smaller quantity of blood contained in a smaller membrane. A membrane approximately 1 cm. in diameter was made, by the usual method, over a piece of glass tubing. It was surrounded by a small water jacket

Table 14. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Infection experiments in chicks to prove that sporozoites are ejected through a membrane on more than one occasion*

1st gorge			2nd gorge			3rd gorge		
Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood	Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood	Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood
39/53 = 74%	4/4	6, 6, 6, 7	21/43 = 49%	2/2	6, 6	—	—	—
114/168 = 68%	2/2	6, 6	59/88 = 67%	4/4	6, 6, 6, 6	29/53 = 55%	3/4	7, 7, 9

inoculated fail to become infected; but failure to infect sometimes occurs when chicks are bitten by heavily infected mosquitoes.

In many chicks the first sign of infection in the peripheral blood was the appearance of minute uninucleate parasites in the erythrocytes, in others schizonts were the first forms to appear, and occasionally mature gametocytes were found before asexual parasites. Adler & Tchernomoretz (1943) have described the appearance of gametocytes

at 40–42° C., and 0·2 c.c. of heparinized chick blood was placed in the membrane tube. Nineteen out of 40 *Aëdes aegypti* gorged through the membrane on the blood, and sporozoites were found in films made from the blood. A little of the blood was diluted in citrate saline solution and injected intravenously into a 9-day-old chick. Parasites were found in the peripheral blood on the 7th day, and death occurred on the 9th day, the infection being very heavy.

Sporozoites were obtained from the same infected

mosquitoes on more than one occasion by membrane feeding (Table 14). The mosquitoes were used for the second or third feed only after the blood from the previous meal was digested, and the proportion which gorged was not significantly less than in previous experiments in which mosquitoes had gorged through a membrane for the first time.

X. SUMMARY

1. Membranes prepared from chicken skin provide a suitable medium through which *Aëdes aegypti* females may be induced to gorge.

2. Under suitable conditions the proportion of female *A. aegypti* which will gorge through membranes, though more variable than when a living chick is offered, is great enough for experimental purposes.

3. It is shown that the gorging reaction in *A. aegypti* is provoked by a heat gradient between the environment and the food-limiting membrane.

4. The feeding reactions of *A. aegypti* towards whole blood, fractions of blood, and other substances have been studied. It was found that (*a*) whole blood, and red corpuscles in saline *when ingested through membranes* go directly into the stomach which becomes fully distended; (*b*) haemoglobin in plasma or distilled water is ingested to a lesser degree than whole blood or red corpuscles in saline, and plasma alone is rarely ingested, but all these pass to the stomach; (*c*) sweet solutions containing glucose or honey are seldom imbibed through membranes and pass to the stomach or diverticula, but only the diverticula are fully distended.

When offered as *open drops* (*a*) blood is seldom ingested, but if ingested passes to the stomach; (*b*) haemoglobin in plasma or water, or plasma alone, are very rarely ingested, but pass mainly to the stomach; (*c*) sweet solutions containing honey or glucose, or mixtures of blood and honey are readily ingested and pass mainly to the diverticula which become fully distended, though traces may be found in the stomach.

5. *Aëdes aegypti* may be infected with *Plasmodium gallinaceum* by allowing them to gorge on drawn infected chicken blood through a membrane. Infection rates comparable to those obtained when the mosquitoes are fed directly on living chickens may be obtained by this method.

6. If infected mosquitoes are allowed to gorge upon uninfected blood through membranes they eject viable sporozoites into the blood. When young chicks are injected intravenously with blood so infected, infections are produced which in period of incubation and intensity are comparable with those resulting from the bites of infected mosquitoes.

7. The ejection of sporozoites through membranes in this manner provides a ready means of obtaining sporozoites free from glandular tissue.

8. Sporozoites collected by this method will be suitable for *in vitro* experiments upon the action of drugs on sporozoites, and also as a source of material for studying in tissue cultures the developmental stages of the malaria parasite arising directly from the sporozoite.

We wish to thank Prof. D. Keilin, F.R.S., and Dr P. Tate for helpful criticism and advice during the course of the work.

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STUDIES ON *ENTAMOEBA HISTOLYTICA*

IV. THE RELATION OF OXIDATION-REDUCTION POTENTIALS TO THE GROWTH, ENCYSTATION AND EXCYSTATION OF *ENTAMOEBA HISTOLYTICA* IN CULTURE

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(With 1 Figure and 9 Graphs in the Text)

INTRODUCTION

The necessity of bacterial flora for the propagation of *Entamoeba histolytica* in culture has long been observed by many investigators. As early as 1926, Dobell & Laidlaw found that *E. histolytica* failed to perpetuate in culture in the presence of a single species of bacterium. In a later paper, Dobell (1931) reported the importance of the presence of certain species of bacteria in culture for the process of encystation of the amoebae. Very recently, great efforts have been made by some investigators to study the influence of the bacterial flora on excystation and growth of amoebae in culture. Snyder & Meleney (1941) observed excystation of *E. histolytica* in bacteria-free cultures when a reducing substance such as cysteine-HCl was added, or when the oxygen tension of the medium was reduced by adding pyrogallol or by bubbling nitrogen gas, but failed to maintain the resulting cultures unless bacteria were added. Chinn, Jacobs, Reardon & Rees (1942) reported that of twenty-six species of bacteria studied, fourteen (*Lept. buccalis*, *Cl. welchii*, etc.) induced excystation and supported growth of trophozoites, while the other twelve, together with three species of yeasts, failed to do so. These authors also stated that rich growth and encystation occurred when the bacterial flora consisted of two or three species of bacteria, especially *Cl. welchii* and *Strept. haemolyticus*, or *Cl. welchii*, *Strept. haemolyticus*, and *Lept. buccalis*. This observation agreed in general with that made by Cleveland & Sanders (1930). Jacobs (1941), in a note, stated that *E. histolytica* grew abundantly in cultures of *Cl. welchii* or *Lept. buccalis*, the oxidation-reduction potential of the former being -500mV. and that of the latter -336mV. The maximal potential at which encystation and positive growth of amoebae were observed was in the range of -114 to -150mV. The length of the period during which a positive culture was maintained was also correlated with the potential in the culture.

Indeed, the preparation of bacteria-free cultures of *E. histolytica* may not be feasible unless the factors that are indispensable for living activities of the amoebae and that are provided by the accompanying bacteria are known. Of possible influential factors, the following appear to be the most important:

(1) The bacterial flora may provide suitable conditions, such as anaerobiosis, *p*H, etc., for the metabolic activity of the amoebae.

(2) The bacterial flora may provide essential food substances for the amoebae.

(3) The bacterial flora may provide enzymic systems for the amoebae in utilizing food material in the medium.

Since the filtrate of cultures of *E. histolytica* failed to support the growth of amoebae (the writer has attempted several times to grow thoroughly washed amoebae—not bacteria-free—in filtrate of 24–48 hr. cultures of strain *NRS* and found the amoebae died in a few hours. The control showed that these washed amoebae were alive), the third factor may not be an important one. The second factor may be important but cannot be determined unless the first is known. Therefore, it is essential to find out how the first factor influences the growth and multiplication of *E. histolytica* in culture.

In the two previous reports (Chang, 1942, 1943), the writer presented the effect of hydrogen-ion concentration on the process of encystation of *E. histolytica* in culture, and the importance of the accompanying bacterial flora in maintaining a favourable *p*H range for encystation as well as for proliferation of the trophozoites. More recently, the writer has studied the oxidation-reduction potential in cultures in its relation to growth, excystation, and encystation. In a series of fifty-five experiments it was revealed that these biological activities were closely related to the oxidation-reduction potential in the culture, and that this was maintained chiefly by the activity of the accompanying bacteria. It is the aim of this paper to present and discuss the results observed in these experiments.

MATERIALS AND PROCEDURE

Three strains of *E. histolytica* (strains *Pt*, *NRS*, and *P*) were used. The cultural histories of the last two strains have been described in the previous reports. The strain *Pt* was prepared by washing

cysts of strain *P* and treating them with fixonal (a surface-active agent), tyrothricin (an antibacterial agent extracted from cultures of *Bact. brevis*, secured through the kindness of Dr R. J. Dubos), and chlorine solution, and inoculating the treated cysts in a culture of *NRS* bacterial flora. This strain behaved in cultures very like strain *NRS*.

The stock cultures were maintained on both liver-infusion-agar slants (modified Cleveland's medium) and egg slants covered with serum-Ringer's solution (in a ratio of 1:10). In studying the potential relations to growth and excystation, the modified Cleveland's medium was used, and in the study of potential relation to encystation, both the fluid encystment medium and a new semi-solid encystment medium consisting of a liver infusion agar slant covered with liver fluid-phosphate-saline-starch mixture buffered at *p*H 7·0–7·1 were used. The preparation of the modified Cleveland's medium and the fluid encystment medium have been described in a previous report (Chang, 1942). Culture tubes 24 × 180 mm. were used for all culture media for potential determinations.

Measurements of oxidation-reduction potential were made with a brilliant platinum wire electrode against a standard calomel half-cell with a saturated KCl-agar bridge connected to a Coleman electro-potentiometer. The *p*H values were determined by a glass electrode. The platinum electrode was made with a 0·02 in. platinum wire and 3 mm. glass tubing filled with mercury. The glass electrode was made with a 3 mm. glass tubing ending in a thin bulb, containing *M*/20 buffered phosphate in iodine and KI solution. The salt-agar bridge was made with an ordinary capillary pipette filled with 2% agar saturated with KCl (sterilized in autoclave). The glass electrode was sterilized in strong chlorine solution and rinsed with sterile distilled water. The platinum electrode was sterilized with strong chlorine solution and then with the wire heated red hot in a flame.

To prepare the culture for determination, a sterile five-hole rubber stopper equipped with the electrodes, salt bridge, one sterile 3 mm. glass tube, 2 in. in length and plugged with cotton (for gas outlet), and one sterile 4 mm. glass tube long enough

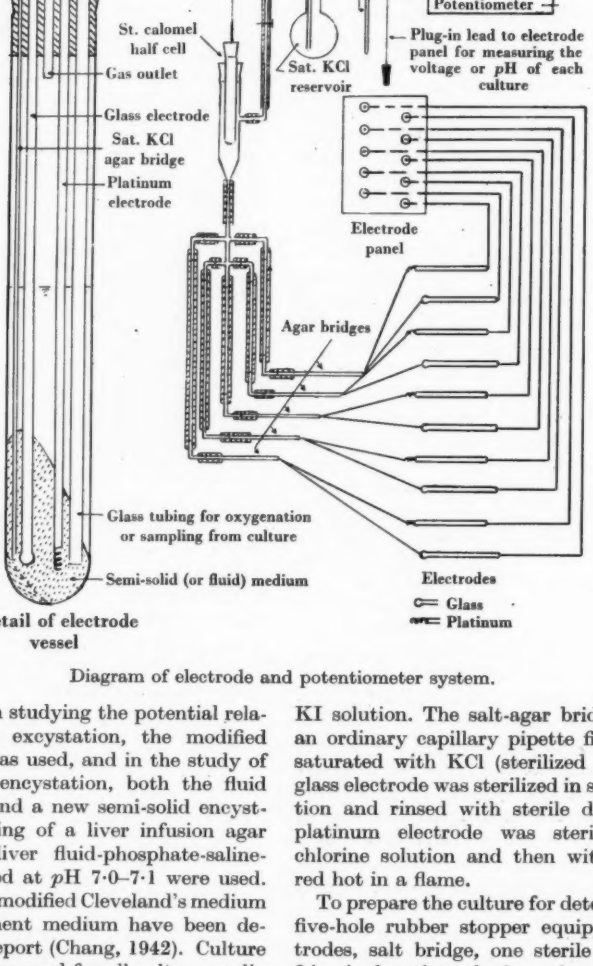


Diagram of electrode and potentiometer system.

to reach the bottom of the culture tube (for taking samples with a fine long capillary pipette, and for gas inlet in case oxygenation was desired). The stopper was fitted to the mouth of a tube of culture medium with the electrodes, salt bridge, and the long glass tube immersed deep in the fluid. The arrangement and connexions are shown in the diagram.

The culture tube was clamped to an iron stand and placed in a water-bath controlled at 37°C. ± 0·3, with the lower half of the tube immersed in water. The potential of the medium was recorded after the medium was warmed up to 37°C. The tube was then planted with the material desired and the potential and *p*H values were determined, and examination of the culture for growth, encystation or excystation was made at scheduled time intervals. When oxygenation was desired, the gas was introduced into the culture through the long glass tube. Contamination was guarded against by passing the gas through a sterilized flask container, containing distilled water and a cotton filter. With the arrangement shown in the diagram, a set of five tubes can be conveniently measured in a single test.

EXPRESSION OF RESULTS

The *p*H values were registered to one-hundredth of a unit. The glass electrode was found accurate to within 0·03–0·05 unit. The oxidation-reduction potentials were expressed as E_h in millivolts calculated from the following equation:

$$E_1 = E_h - E_{st}, \text{ or } E_h = E_1 + E_{st},$$

where E_h is the potential with reference to the hydrogen electrode, E_1 the potential observed on the potentiometer, and E_{st} the potential of the standard calomel half-cell with saturated KCl which is about 238mV. at 37°C.

Since numerical determinations of the increase of number of amoebae could not be made accurately without interfering with the potential determinations, the richness of the growth was determined by the number of amoebae per field under low power in a drop of the sediment taken from the culture and indicated by +, + +, + + +, + + + + signs. One plus means slight increase in growth, + + moderate increase in growth, + + + great increase in growth, and + + + + tremendous increase in growth. These signs were also used to indicate the richness of cyst yield. One plus indicates less than 10% amoebae encysted, + + 10–25% encysted, + + + 25–40% encysted, and + + + + 40–65% encysted.

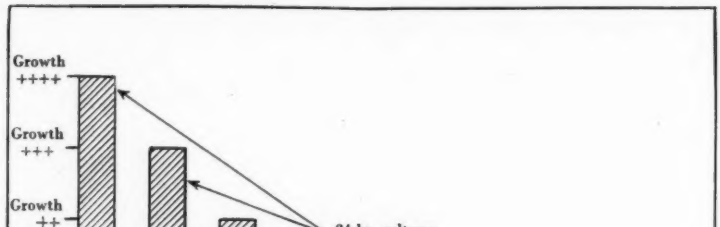
Experiments on relation of oxidation-reduction potentials to growth of amoebae

Tubes of modified Cleveland's medium were planted with well-mixed, uniformly sized inocula containing trophozoites. The oxidation-reduction potentials of these cultures were maintained at a more or less fixed level for a period of about 18 hr. by bubbling oxygen gas at different rates, with a fluctuation of potentials within 25mV. The lowest potential was maintained by leaving the culture alone. Several cultures maintained at high potentials were also prepared by washing the amoebae thoroughly with sterile normal saline before inoculating into the medium. In this case, controls were always made to insure that these washed amoebae were alive. Results obtained from thirty cultures with oxygenation and four with washed amoebae are presented in Graph 1. From this graph it is learned that, under otherwise similar conditions, trophozoites proliferated most prosperously at a range of potentials of from −350 to −425mV. The rate of proliferation was less at −275 to −350mV. and still less at −200 to −275mV. There was a bare increase of amoebae at −150 to −200mV., and above −150mV. there was no evidence of multiplication. At potentials of +50 to −50mV. the amoebae were alive for 12–24 hr., and at +100 to +200mV. they disintegrated in 1 or 2 hr.

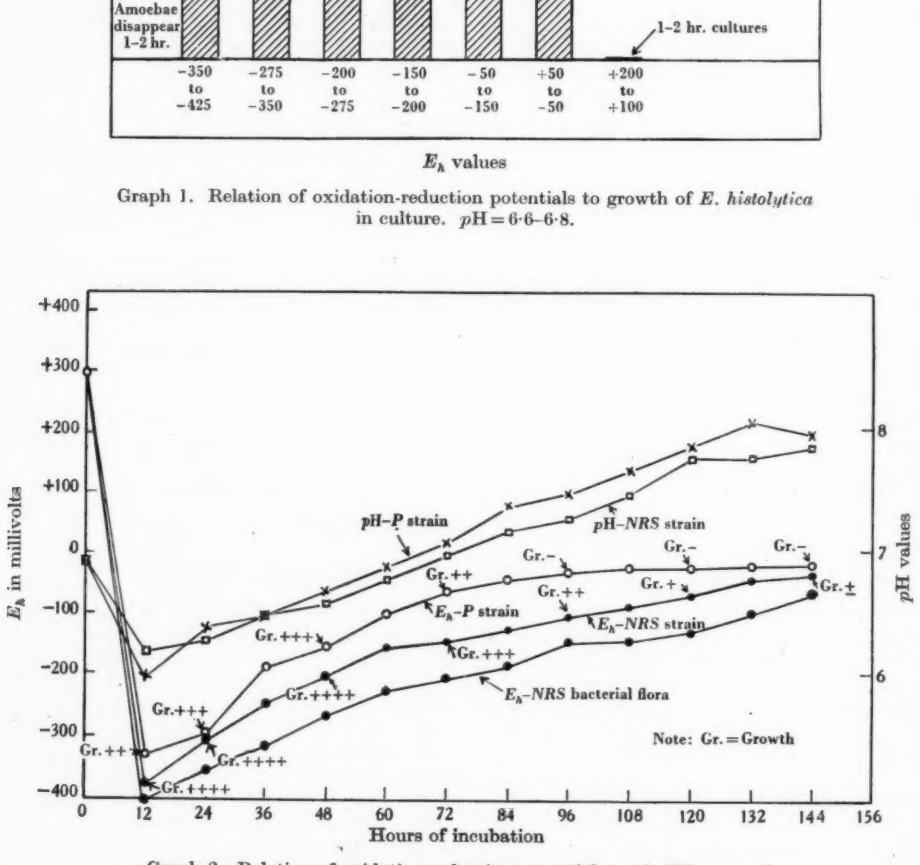
It is of interest to note that the average size of the trophozoites observed in cultures at low potentials (below −300mV.) was 3–4 μ smaller than of that observed in cultures at higher potentials (between −200 and −250mV.)—the average diameter of the former being 18 μ and that of the latter 21–22 μ. At potentials between −80 and −160mV., especially at low *p*H values (between *p*H 5·5 and 6·5), the trophozoites were often very large, some were about ten times as big in area as those at potentials below −300mV. It was thought that under very low potential conditions the proliferation of amoebae was so fast that their size decreased considerably.

These observations seem to indicate that *E. histolytica* is a protozoan which proliferates only under strictly anaerobic conditions. At potentials above −150mV. reproduction seems to be interfered with, and at potentials above −50mV. the metabolic activity of the amoebae seems retarded and they die and disintegrate within 12 hr. or more.

In order to find out whether the life span of a culture of *E. histolytica* is associated with the oxidation-reduction potential in the culture, eight tests (thirty-two cultures) were made with two strains, *P* which required a transfer every 2 days, and *NRS* which maintained a good growth with transfer every 5 days. Though we were working with cultures containing an unknown number of unknown bacteria, the results observed in different tubes were surprisingly consistent, and the potentials observed in different cultures rarely exceeded a standard deviation of 12mV. The results obtained are presented in Graph 2, where it is shown that the life span of a culture of amoebae was also related



Graph 1. Relation of oxidation-reduction potentials to growth of *E. histolytica* in culture. *p*H = 6·6–6·8.



Graph 2. Relation of oxidation-reduction potentials to the life span of a culture of *E. histolytica*.

to its oxidation-reduction potential. In cultures of strain *P*, which showed a fair or good growth only in the first 2 days of incubation, the potential was below −150mV. also only in the first 2 days. After the third, the potential was around −50mV. and the growth ceased. On the contrary, the culture of strain *NRS*, which showed a fair growth even on the fifth day of incubation, did not have such an unfavourable potential until the sixth day. The *p*H values observed in cultures of both strains differed by 0·1–0·2 unit—the *p*H values in cultures of strain *P* being about 0·2 unit lower than those in cultures of strain *NRS* in the first 12 hr., and 0·1–0·2 unit higher during the rest of the period of incubation. Therefore it seems apparent that the life span of a culture of *E. histolytica* depends, chiefly if not entirely, on how long the oxidation-reduction potential in the culture may be maintained low enough to permit growth of the amoebae, provided, of course, there is enough food supply for the activity of both amoebae and bacteria.

Experiments on the relation of oxidation-reduction potentials to excystation

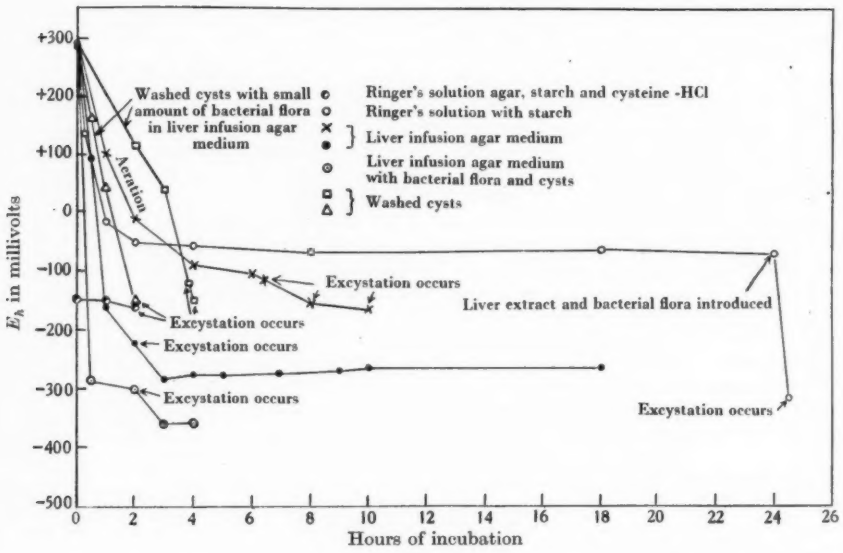
A total of nine tests (thirty-six cultures) has been made with culture-induced cysts of both strains *Pt* and *NRS* in the modified Cleveland's medium, Ringer's solution with starch, and Ringer's solution with starch, agar and cysteine-HCl. The agar was used to prevent diffusion of oxygen into the deeper part of the medium and was found quite effective. In some cultures, oxygenation was applied to control the oxidation-reduction potential at some fixed levels. The results thus observed are shown in Graph 3. It is clearly indicated in this graph that, no matter what media are used, the process of excystation of *E. histolytica* takes place only when the potential of the culture is dropped to −115mV. or lower. It has also been repeatedly observed that proliferation of the metacystic amoebae did not occur until the potential had dropped to −150mV. or lower. In tubes in which the potential was maintained at above −115mV. for 32 hr. or more, excystation failed to occur even when the potential dropped to −120mV. or lower, due apparently to the fact that cysts were viable only for about 32 hr. at 37°C. This difference in potential levels for excystation and multiplication may explain why conditions which permit excystation may not support the growth and multiplication of the amoebae.

Experiments on the relation of oxidation-reduction potentials to encystation

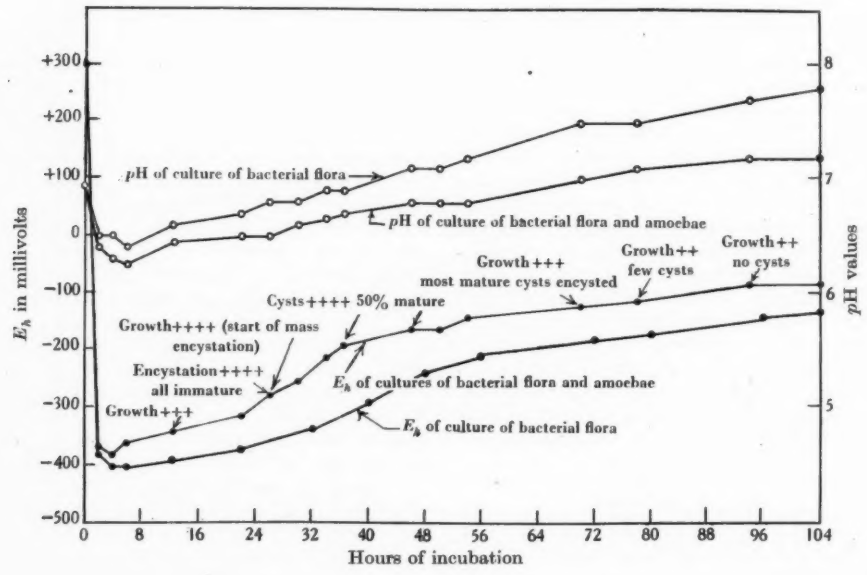
Twenty-eight tests have been made to study the relation of potentials in the culture to encystation. The procedure of inducing encystation was essentially the same as that described in a previous report (Chang, 1942). Averages of the results (which had a standard deviation of +15mV.) are presented in Graphs 4–9. Graph 4 was based on observations made with strain *NRS* in the fluid encystment medium, Graph 5 with strain *NRS* in the semi-solid encystment medium, and Graph 6 with strain *Pt* in the semi-solid medium. In these three graphs it was noticed that the potentials dropped sharply to below −300mV. 30 min. after the inoculation. This lack of a lag period which was so frequently observed in ordinary bacteria cultures was most probably due to the huge size of inocula used here (1–1½ ml. of sediment from cultures in Drbohlav's medium). The lowest potential was reached in about 6 hr. of incubation, which was about −380mV. with *NRS* in the fluid encystment medium, −425mV. with *NRS* in the semi-solid encystment medium, and −380mV. with *Pt* in the semi-solid medium. The potential then rose gradually and reached about −300mV. after 22–24 hr. After this period of gradual rise of potentials there followed a period of 10–16 hr. in which the potential rose more sharply. This 'sharp rise of potential' is of great interest and importance in the process of encystation, because it was at the beginning of this period that mass encystation occurred, and during this period that maturation of cysts took place. In these three graphs it is also noticed that more abundant cysts were produced in the new semi-solid encystment medium in which the potential remained low (below −300mV.) for more than 20 hr. than in the fluid encystment medium in which the potential remained below −300mV. for only about 14 hr. This long period of low potentials preceding the 'sharp rise of potential' permitted a greater multiplication of the trophozoites and, consequently, a larger cyst yield. When mass encystation started, it was found that these newly formed cysts occurred in crops, indicating that they were encysted from trophozoites that grew in patches resulting from rapid multiplication. Hence it seems that it is chiefly those trophozoites having undergone a rapid multiplication that encysted.

The occurrence of mass encystation at the 'sharp rise of potential' after a period of low potentials may be explained by the change of a more favourable to a less favourable condition. As stated before, the trophozoites proliferated at a tremendous rate during this low potential pre-encystation period. As the potential rose more sharply, the condition became less favourable for the same amount of activity and the trophozoites encysted.

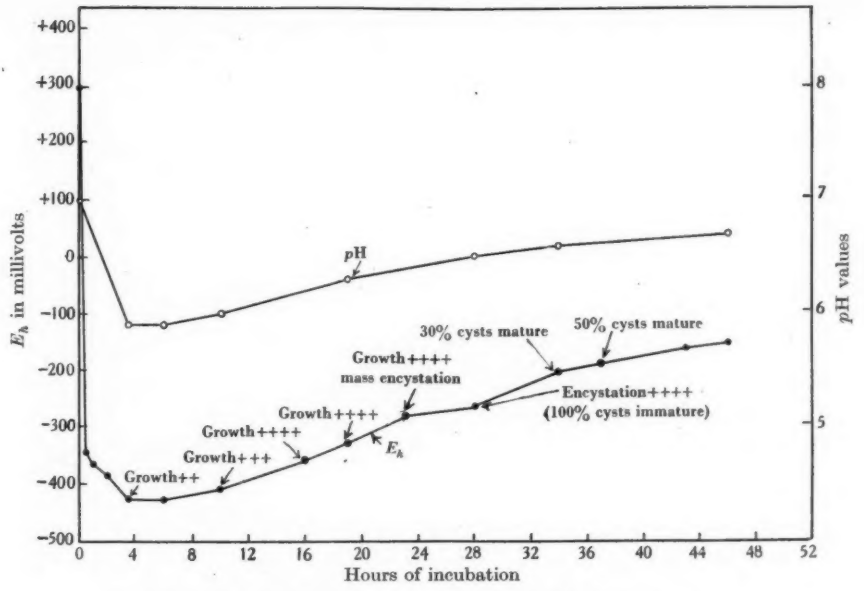
However, it must also be noted that this 'sharp rise of potential' did not always induce encystation. Many cultures have been prepared with trophozoites long maintained in the modified Cleveland's medium, in which the rate of multiplication had been constantly high, and no mass encystation was observed although this 'sharp rise of potential' was



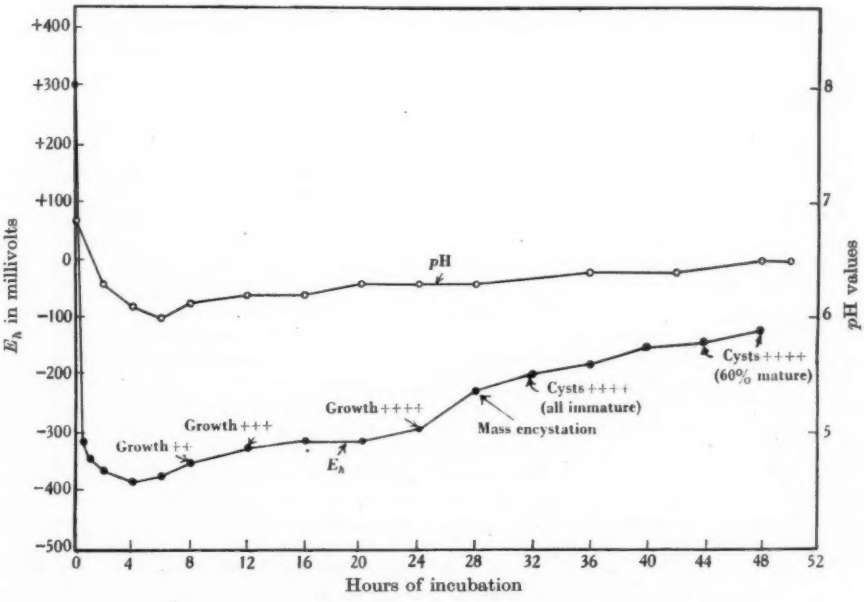
Graph 3. Relation of oxidation-reduction potentials to excystation of *E. histolytica* in culture.



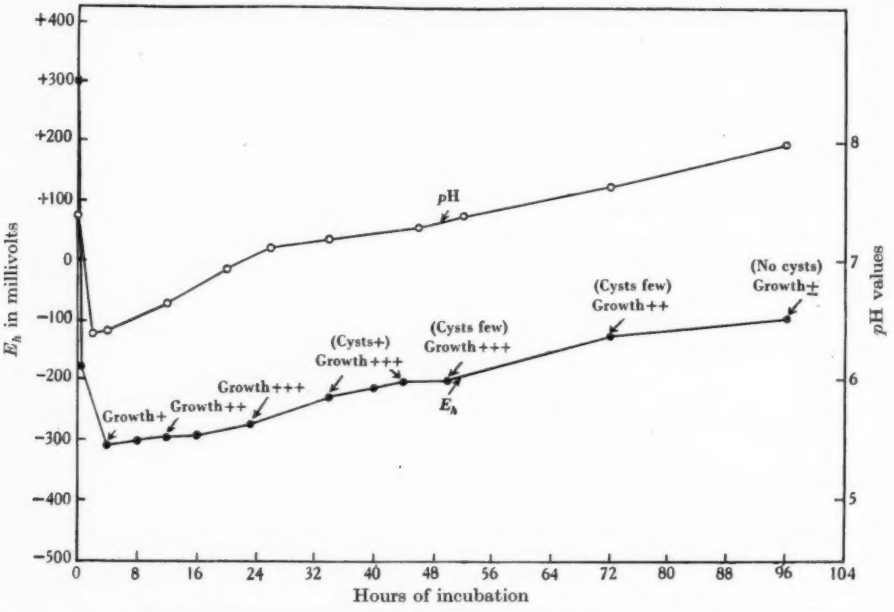
Graph 4. Oxidation-reduction potentials and hydrogen-ion concentration changes in a culture of *E. histolytica* and a culture of bacterial flora alone.



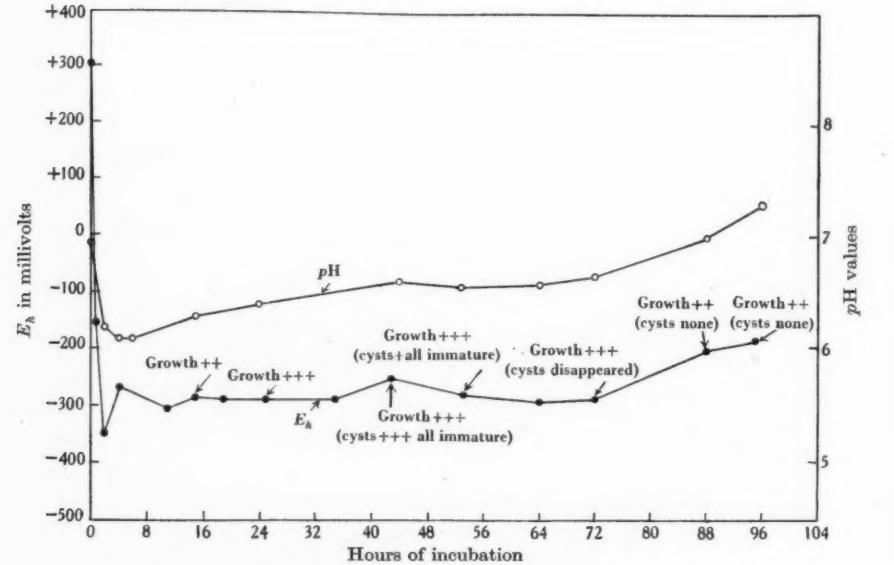
Graph 5. Relation of oxidation-reduction potentials to encystation of *E. histolytica* in culture. *p*H = 5·9–6·7.



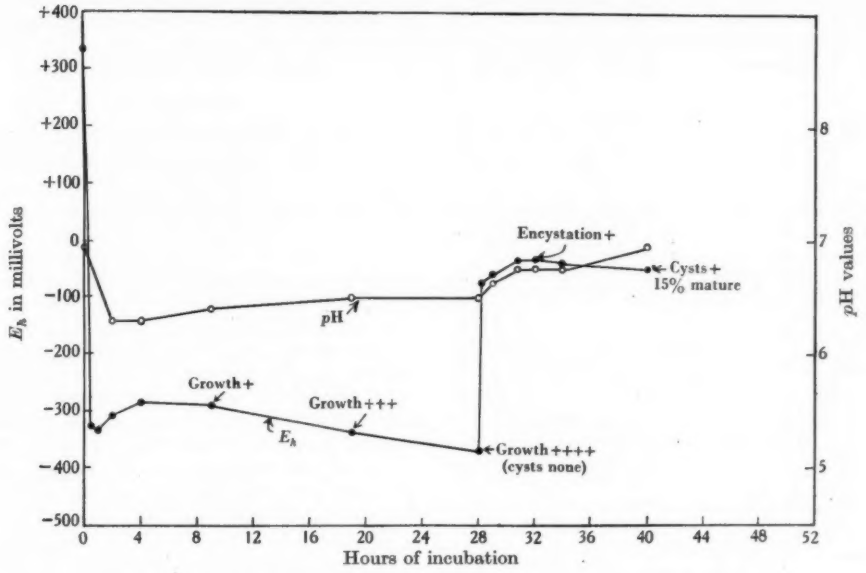
Graph 6. Relation of oxidation-reduction potentials to encystation of *E. histolytica* in culture. *p*H = 6·0–6·5.



Graph 7. Relation of oxidation-reduction potentials to encystation of *E. histolytica* in culture. pH = 6·4–8·0.



Graph 8. Relation of oxidation-reduction potentials to encystation of of *E. histolytica* in culture. pH = 6·1–7·3.



Graph 9. Effect of oxygenation on encystation of *E. histolytica* in culture.

always there. This indicates that there must be an accelerated rate of multiplication before the trophozoites can be induced to encyst. There are also, as stated in a previous paper (Chang, 1942), strains of amoebae that have been maintained very long at a constant high rate of multiplication and that have lost the tendency to encyst, and encystation may not occur even if these factors necessary for encystation are present.

It is also of interest to note that those cysts induced in cultures having a very low potential in the first 16–22 hr. of incubation and a very high rate of multiplication, were definitely a few microns smaller in the average diameter than those induced in cultures where the potentials in the pre-encystation period were not as low and rate of multiplication was not as high. This is apparently because the average size of trophozoites was smaller in the former than in the latter.

The *p*H values presented in these three graphs showed the same pattern as those reported in the previous paper in that the *p*H dropped sharply in the first 6 hr. of incubation and then came up slowly throughout the rest of the incubation period. On the whole, the *p*H values were more or less parallel to the oxidation-reduction potentials.

In order to show the relation of *p*H to encystation, two tests (totalling ten cultures) were made with encystment medium prepared at *p*H 7·4. The results are shown in Graph 7. In this it is shown that the 'sharp rise of potential' which occurred after about 24 hr. of incubation was not as sharp as that observed at lower *p*H values, and that mass encystation failed to occur. Whether this was due to the lack of a sharper rise of potential after a high rate of multiplication was reached or to the fact that the trophozoites were less affected by the change of potentials at higher than at lower *p*H values was not determined. Probably both played a part. If the second factor was true, the *p*H effect on potential requirement of amoebae would be just opposite to that on anaerobic bacteria, which seem to tolerate a higher potential better at lower than at higher *p*H values. It must also be noted that the *p*H values at which good encystation was observed covered a lower range, 6·4–6·8, than those reported previously, 6·8–7·1 (Chang, 1942).

Since it had been observed that in cultures of strain *P* mass encystation failed to occur, and at other times mass encystation occurred but the cysts failed to undergo maturation and disappeared in 12 hr. of incubation or more, attempts were made to find out whether these phenomena were related to the potential changes. In twelve cultures (Graph 8) it is revealed that although mass encystation did occur at the 'sharp rise of potential', nevertheless at later hours of incubation than that observed with strains *Pt* and *NRS* the potential went down again in a few hours' time and maturation of cysts failed to occur. Therefore, it would

seem that maturation of cysts took place if the 'sharp rise of potential' was maintained.

After having observed these potential changes in relation to encystation, attempts were made to induce mass encystation and maturation of cysts with strain *P* by artificially changing the potential by means of oxygenation. In a total of twelve cultures prepared, seven showed negative results. The result of one of the other five that showed encystation is presented in Graph 9. From this it is learned that, although encystation and maturation of cysts did take place when the 'sharp rise of potentials' was artificially induced, the percentages of encystment and mature cysts were not nearly as rich as those under natural conditions. It was also found that, in those cultures in which encystation occurred, there was a high rate of multiplication of trophozoites in the pre-encystation period, and the *p*H value was in the range of 6·5 and 6·8. This again shows that factors influencing encystation are many and a mere change of the potential in the culture may not induce encystation.

Experiments on the relation of bacterial flora to oxidation-reduction potentials

In order to show how important the bacterial flora is in maintaining the potential in the culture for the growth activity of the amoebae, cultures of the bacterial flora isolated from a culture of *E. histolytica* (strain *NRS*) have been studied. The results observed in eight cultures in the modified Cleveland's medium are presented in Graph 2 and those observed in eight cultures in the semi-solid medium are presented in Graph 4 to show the differences in potentials between cultures of the bacterial flora with and without the amoebae. In these two graphs we observed that the potentials in the culture of bacteria alone were always 30–60mV. lower than in those of bacteria and amoebae. This certainly indicates that the low potential in a culture of *E. histolytica* is maintained chiefly by the activity of the accompanying bacteria.

It is of interest to note that the 'sharp rise of potentials' in cultures of amoebae and bacteria was apparently not observed in those of bacteria alone. The trends of potential changes observed in the mixed cultures as well as in cultures of bacteria alone seem to indicate that the bacterial activities were greatest in the first 12 hr. of incubation, and that, as the growth of amoebae became rich or abundant, the bacterial activities became more or less depressed as reflected by the gradual rise of potentials after 12 hr. When the depressing effect was great, the 'sharp rise of potentials' occurred. The observations of Chinn *et al.* (1942) that *Lept. buccalis* grew more richly in pure cultures than in those mixed with amoebae, and that in cultures mixed with amoebae the number of bacteria decreased as the number of amoebae increased, seem to support this assumption.

DISCUSSION

It is well known that bacterial cultures are usually strongly reducing systems that result from the metabolic activity of the bacteria. Utilization of these reducing systems in cultures of aerobic bacteria for cultivation of anaerobic bacteria under an aerobic condition has also been successfully tried out. The necessity of bacteria for the propagation of *E. histolytica* has been recognized ever since this organism was cultivated in artificial media. However, the interpretation that the bacteria furnished reducing systems for the proliferation of amoebae has not been definitely made. In the present study, it is shown that this pathogenic intestinal protozoan is apparently a strictly anaerobic organism, requiring a strongly reducing state for its metabolic activity, and that this reducing state is provided by the accompanying bacteria.

The fact that not all species of bacteria can support the growth of *E. histolytica*, as observed by Cleveland & Sanders (1930) and Chinn *et al.* (1942), is of great interest and may, at least in part, be explained on the basis of oxidation-reduction potential requirement. For instance, the single species of bacteria that supported best the growth of amoebae, as shown in the latter report, were *Lept. buccalis* and *Cl. welchii*, while those like *B. coli*, *Sal. paratyphi*, yeasts, etc., failed to do so. The potentials in the culture of *Lept. buccalis* and *Cl. welchii*, as given by Jacobs (1941), were −336 and −500mV. respectively. Hewitt (1936) gives the potential in the culture of *Sal. paratyphi* as −48 to −93mV. and that in cultures of yeast +90 to +270mV. The potential in the culture of *B. coli* as given by Hewitt is as low as −400mV., but this low potential remains only in the first few hours of incubation and it goes quickly to +100mV. after 12 hr., and +400mV. after 30 hr. From these potentials quoted, it is understandable why some bacteria supported the growth of amoebae, and some failed to do so.

While the writer brought out the close relation of the oxidation-reduction potential and the growth activity of *E. histolytica*, he had no intention of neglecting the other possible factors. However, he feels that the importance or unimportance of the other factors could be determined by finding if *E. histolytica* would propagate in cultures in which a strongly reducing condition (below −200mV.) was provided without the presence of bacteria.

The maximal oxidation-reduction potential at which excystation ever took place was in the range of −115 to −125mV. The maximal potential for proliferation of trophozoites was in the range of

−150 to −170mV. Hence a potential which is sufficiently low for encystation may not support proliferation. Furthermore, it is the writer's impression that, in cultures of *E. histolytica*, if the potential was maintained between −150 and −200 mV., only a small percentage of the trophozoites multiplied and the rate of multiplication was slow, and, in the long run, such cultures generally died out. Therefore, it seems that in order to propagate a strain of amoebae indefinitely in culture, the potential condition should be sufficiently low for favourable proliferation.

The occurrence of mass encystation at the 'sharp rise of potentials' in the encystment culture of *E. histolytica* indicates that the process of encystation is a response on the part of amoebae to a rather sudden change of the environment from a more favourable to a less favourable condition. The fact that encystation only happened in those cultures wherein the trophozoites had been undergoing an accelerated rate of multiplication indicates that, besides the external factor, there is also an internal factor involved. The greater the increased rate of multiplication, the greater the tendency to encyst. Therefore it seems that a so-called encystment medium is nothing more than one which contains a rich supply of reducing substances favouring abundant multiplication. However, such medium may not be successful if the bacterial flora is not capable of producing such a reducing condition in the pre-encystation period and the 'sharp rise of potentials' after multiplication has reached its peak.

Attempts have been made to prepare bacteria-free cultures of amoebae in a cellophane bag immersed in a bacterial culture. Cysts were thoroughly washed and treated with fixanol, tyrothricin, and chlorine, and inoculated in the bag containing liver infusion agar medium (sterilized in autoclave). In some trials all cysts were found killed. In other trials, encystation and a rich growth of amoebae were observed. Unfortunately, in all those bags in which positive cultures were obtained, bacteria began to appear after 2–3 days of incubation. Nevertheless, it was noticed that the number of bacteria present in the bag in the early stages of incubation was so small as compared with the growth of trophozoites that it seemed unlikely that the bacterial growth had much to do with the growth of amoebae. The potential in the bag in the first 2 days of incubation was found (in two cultures) in the range of from −300 to −200mV. and was about 30–50mV. higher than that in the bacterial culture outside. Positive cultures were maintained in several transfers from bag to bag; but, if the content in the bag was inoculated in a tube of modified Cleveland's medium, the amoebae died out in 12–24 hr., indicating that the bacteria in the bag were not able to support the growth of amoebae. It is still hoped to prepare bacteria-free cultures of amoebae by this technique.

SUMMARY AND CONCLUSION

1. The oxidation-reduction potential in cultures of *E. histolytica* has been studied. It was found that a close relation exists between the potential in the culture and the growth, encystation, and excystation of *E. histolytica*. The small differences observed in different cultures indicate the reproducibility of the potentials and the reliability of the potential condition in explaining these biological activities of the amoebae in culture.

2. The best growth of trophozoites was observed at potentials between −350 and −425mV., less at potentials between −275 and −350mV., still less at between −200 and −275mV. At potentials between −150 and −200mV. a bare increase in number of trophozoites was noticed. Between potentials of −50 and −150mV. no increase of growth was observed. Trophozoites were found dead in 12 hr. or more at potentials between −50 and +50mV., and died and disintegrated in a couple of hours between potentials of +100 and +200mV.

3. The life span of a culture of *E. histolytica* was also found to be related to the potential condition. The longer the potential in the culture remains below −150mV., the longer the culture remains positive.

4. Excystation of *E. histolytica*, no matter in what media, took place only at potentials of −115mV. or lower.

5. Mass encystation of *E. histolytica* was induced in cultures in which a 'sharp rise of potential' occurred after a greatly increased rate of multiplication of trophozoites after 22–24 hr. of incubation. Maturation of cysts occurred during this period of 'sharp rise of potentials'. If the potential went down shortly after its sharp rise, the cysts failed to become mature and disappeared in about 12 hr.

6. Trophozoites and cysts observed in cultures with very low potentials (below −300mV.) and a rapid multiplication were definitely smaller than those in cultures having higher potentials and trophozoites multiplied slowly.

7. In cultures of amoebae in which mass encystation failed to occur, or mass encystation occurred but cysts failed to become mature, some success was observed in a few trials by artificially creating the 'sharp rise of potential' by oxygenation.

8. The *p*H values noticed in cultures at times of encystation ranged from 6·4 to 6·8. This *p*H range was a little lower than that observed previously. Mass encystation failed to occur at the change in potential if the *p*H was 7·2 or higher.

9. It is felt that the process of encystation requires, at least, two extrinsic and one intrinsic factors. The first two are the potential change from

more favourable to less favourable conditions and the *p*H. The last one is the tendency to encyst possessed by the amoebae.

10. The low potential condition in a culture of *E. histolytica* was found chiefly provided by the activity of the accompanying bacteria. It is, therefore, felt that the indispensability of a bacterial flora for cultivation of amoebae may be explained, partly if not entirely, by the potential relation. To verify this statement, it is hoped to propagate *E. histolytica* in cultures in which a strongly reducing condition is provided without the presence of bacteria.

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